Studies on the phosphorylation of pyridine coenzymes in microorganisms(Dissertation_全文)

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STUDIES ON THE PHOSPHORYLATION OF PYRIDINE COENZYMES IN MICROORGANISMS

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ABBREVIATIONS

NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NMN	Nicotinamide mononucleotide
NAm-R	Nicotinamide riboside
NAm	Nicotinamide
Ad-R	Adenosine
AMP	Adenosine monophosphate
AR-diP	Adenosine 2'(3'),5'-diphosphate
ATP	Adenosine triphosphate
ADPR	Adenosine diphosphate ribose
GMP	Guanosine monophosphate
GR-diP	Guanosine 2'(3'),5'-diphosphate
IMP	Inosine monophosphate
HR-diP	Inosine 2'(3'),5'-diphosphate
UMP	Uridine monophosphate
UR-diP	Uridine 2'(3'),5'-diphosphate
CMP	Cytidine monophosphate
CR-diP	Cytidine 2'(3'),5'-diphosphate
UDPG	Uridine 5'-diphosphoglucose
p-NPP	*p*-Nitrophenyl phosphate
p-NP	*p*-Nitrophenol

INTRODUCTION

The presence of pyridine nucleotide coenzymes, the biologically active forms of nicotinic acid or nicotinamide, was first suggested by Harden and Young.[1] They discovered that some dialyzable and heat stable substance present in the cell-free extract of brewer's yeast was necessary for the fermentation of glucose. The chemical structure of this substance, named Coenzyme I (NAD) later, was taken up by many investigators and was well demonstrated by means of chemical and enzymic techniques (Formula 1).[2-4] On the other hand, Warburg and Christian[5] isolated another coenzyme (Coenzyme II, NADP) which is required for the oxidation of glucose 6-phosphate to 6-phosphogluconate. The structure of this coenzyme was clarified by many investigators to have the similar composition to that of NAD except for the presence of the third phosphoryl group in the adenosine moiety as shown in Formula 1.[6,7]

Conversion of NAD to NADP, phosphorylation of NAD, is catalyzed by NAD kinase.[8] This reaction is the only known pathway of NAD phosphorylation and distributes in a variety of organisms. NADH is also phosphorylated to NADPH by the enzyme obtained from yeast, whereas plant enzyme is unable to catalyze this reaction.[9] Yamamoto[10] and Kumada[11] indicated that redox states of NAD might control the level of NADP in plant tissues; under anaerobic conditions, the oxidation of NADH was delayed where the inhibition

of NAD kinase took place. Recently, a possibility of regulation of NAD kinase by NADPH and NADP in animal tissues was suggested.[12]

The present study is concerned with the phosphorylation of NAD by microorganisms and with the distribution of the reaction. In the course of the investigation, the author found that NAD was phosphorylated by a new phosphotransferring reaction other than the reaction of NAD kinase. It was also found that this new phosphotransferring system was limited only in some microorganisms, whereas NAD kinase activity distributed widely in a variety of microorganisms. Furthermore, the enzymic properties of the phosphotransferase preparation were discussed.

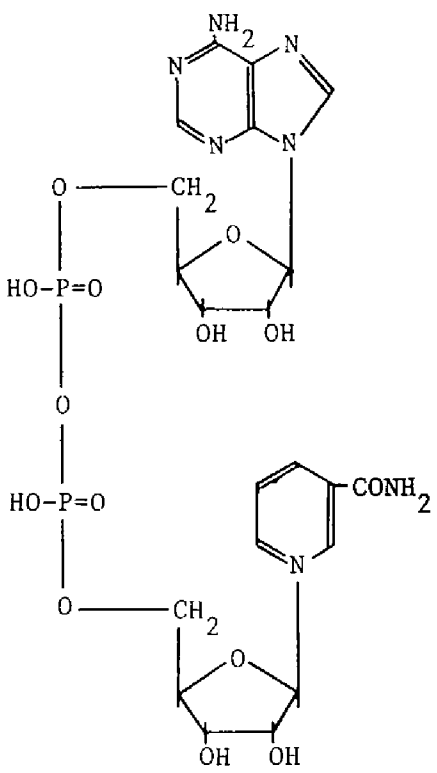
An enzyme system responsible for NAD biosynthesis from nicotinic acid or nicotinamide was detailed by Preiss and Handler[13,14] and other investigators.[15] This pathway has been found to occur in a number of organisms.[16-18] Nishizuka and Hayaishi[19-21] studied other pathway from tryptophan via quinolinate in animal tissues. The presence of *de novo* synthetic pathway of the pyridine coenzymes, in which aspartic acid and glycerol or their derivatives are the most likely condensing partners, is proposed by Hadwiger *et al.*,[22] Chandler *et al.*[23] and Isquith and Moat.[24] Microorganisms seem to contain more than one biosynthetic pathway of NAD and the pathway given above might be regulated by coenzyme levels and physiological conditions.

In yeast, the tryptophan pathway operates under aerobic conditions, whereas the *de novo* pathway becomes predominant under the conditions in which the tryptophan pathway does not function.[25)]

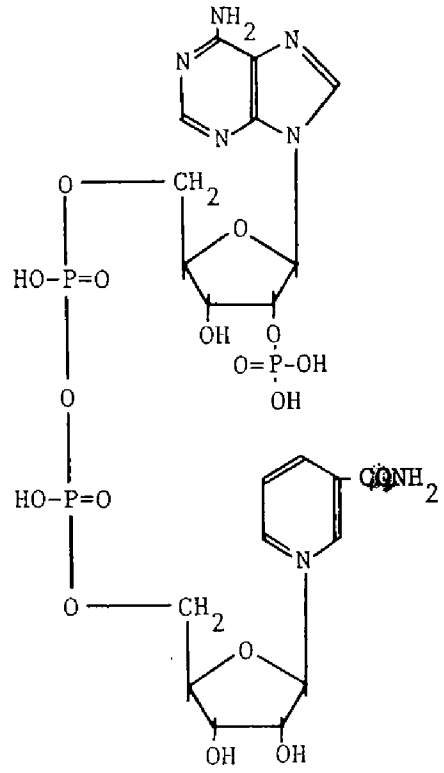
In biological systems, several nicotinamide derivatives besides NAD, NADP and NMN are involved. In the molecule of biologically active NAD or NADP, the configuration of the glycoside bond of nicotinamide riboside moiety is in the β-form. However, a small amount of α-isomers of NAD and NADP are present naturally besides large amounts of β-isomers.[26)] Deamido-NAD and deamido-NMN, precursors of biosynthesis via Preiss and Handler pathway, and their α-configuration forms are also contained in bacterial cells.[27,28)] Some microorganisms such as yeast and *Proteus* contain Coenzyme III (nicotinamide riboside 5'-pyrophosphate).[29)]

Recently, Imai *et al.*[30)] isolated from cells of *Azotobacter vinelandii*, a new pyridine nucleotide, adenosine-diphosphoribosyl-NAD, in which the ADP-ribose is glycosidically linked to NAD at position 2' or 3' of the nicotinamide mononucleotide moiety.

Nishizuka *et al.*,[31,32)] Chambon *et al.*[33)] and Sugimura *et al.*[34)] concurrently reported that a particulate fraction obtained from mammarian nuclei was capable of incorporating the adenosine diphosphate ribose moiety of NAD into an acid-insoluble material, synthesizing homopolymer of ADP-ribose. The function of this

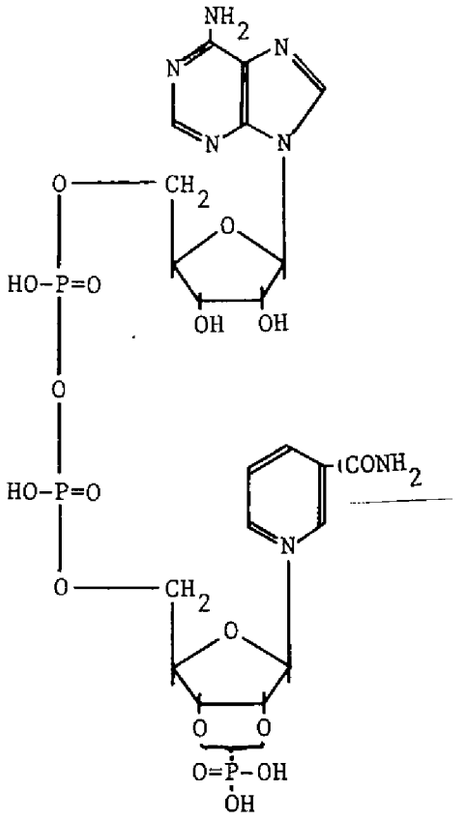


NAD

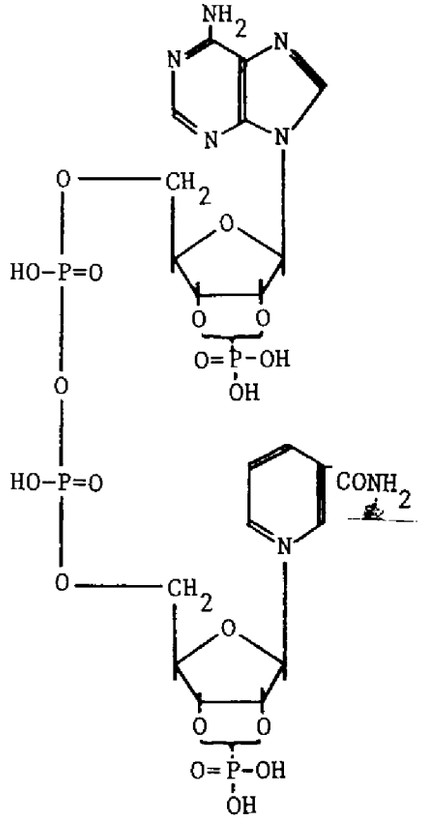


NADP

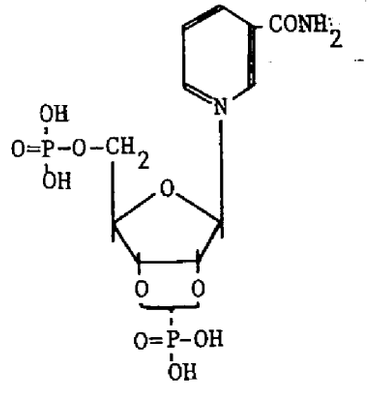
Formula 1. Chemical Structure of Pyridine Coenzymes.



NADP analog (Compound I) (phosphorylation product of NAD)



NAD diphosphate (Compound V or VI) (phosphorylation product of NADP or NADP analog)



Nicotinamide riboside diphosphate (Compound III or IV) (phosphorylation product of 5'-NMN or degradation product of NADP analog or NAD diphosphate)

Formula 2. Chemical Structure of New Pyridine Nucleotides.

substance has been obscure yet.

Various pyridine nucleotide analogs have been found to be synthesized with the aid of enzymic and chemical reactions. It was demonstrated by Kaplan *et al.*[35,36] that NADase of pig brain catalyzed exchange reactions between various substituted pyridine compounds and nicotinamide moiety of NAD to form various analogs of NAD, and some of them showed coenzyme functions on typical dehydrogenases.

In the present study, it was shown that the NADP analog, of which chemical structure was different from that of NADP, was formed besides NADP by the new phosphorylating reaction of NAD. Furthermore, pyridine nucleotides such as NADP, NMN and NADP analog as well were phosphorylated to form new pyridine nucleotides. Chemical structure of these compounds are summarized in Formula 2. It is an outstanding feature that these compounds contain a phosphoryl group in the nicotinamide riboside moiety.

One of the most important functions of pyridine coenzyme is their participation in biological oxidoreduction systems. Pyridine nucleotides are reversibly converted to reduced forms in the presence of suitable hydrogen donors and enzymes, dehydrogenases. Mechanisms of the reduction of pyridine coenzymes were elucidated with the aid of various kinds of chemical re-

ducing agents and dehydrogenases. In the reduction of the pyridine ring of these coenzymes, two equivalents of hydrogen per mole are required.[37,38] In the biological systems, there operate numerous oxidation-reduction processes and the pyridine coenzymes are involved in a variety of metabolic pathways such as glycolysis, tricarboxylic acid cycle, pentose phosphate cycle, and other biosynthetic and catabolic metabolisms.

Recently, regulatory mechanisms involving pyridine coenzymes have been reported. It was suggested that in *Escherichia coli* the level of NADPH was the central control signal for coordinating the activity of a variety of enzymes which utilize oxaloacetate or malate as substrates. Malate dehydrogenase,[39] NADP-specific malic enzyme,[40] citrate synthetase[41] and phosphoenolpyruvate carboxylase[42] were found to be inhibited in an allosteric manner by NADH. ADP-glucose pyrophosphorylase of *E. coli*[43] and ribulose 5-phosphate kinase in *Rhodopseudomonas spheroides*[44] were activated in an allosteric manner.

Recent progresses in studies of pyridine coenzymes have elucidated that these coenzymes possess biological functions other than those mentioned above. Gellert[45] and other investigators[46,47] reported that NAD took part in an enzymic reaction of repairing an interrupted strand in a DNA duplex. In this reaction NAD functioned as the adenosyl moiety donor toward the enzyme

protein.

Generally speaking, new pyridine nucleotides obtained in this experiment showed no or only a slight activity for several dehydrogenases. Whether these nucleotides are contained in biological systems and play some biologically active roles are problems to be discussed.

Chapter I. Phosphorylation of Pyridine Nucleotides by a New Phosphotransferring System

Section 1. Phosphorylation of NAD

INTRODUCTION

The phosphorylation of NAD to NADP is catalyzed by NAD kinase and this reaction has been known to be the sole pathway of NADP biosynthesis. This enzyme has been found in several biological systems such as mammalian and avian tissues and microorganisms. Properties of the enzyme have been studies in detail by many investigators.[8,48,49] In this enzyme reaction, the phosphoryl donor is strictly limited to ATP.

Recently, Mitsugi *et al.*[50-52] reported the phosphorylation of nucleoside or nucleoside monophosphate by bacterial nucleoside phosphotransferase other than by nucleoside kinase and Ogata *et al.*[53] observed the phosphorylation of pyridoxine by a new phosphotransferring enzyme different from pyridoxine kinase in several microorganisms.

The author observed that when NAD and organic phosphates such as *p*-NPP or nucleoside monophosphate were incubated with the enzyme preparation of *Proteus mirabilis* (IFO 3849), NADP and an unidentified compound were formed in the reaction mixture. In this section, conditions for this enzymic phosphorylation of

NAD are discussed and the isolation of NADP is described.

MATERIALS AND METHODS

Cultivation of bacteria and preparation of enzyme solution. *Proteus mirabilis* (IFO 3849) used in this investigation was grown in a liquid medium of the following composition: glucose, 2.0%; peptone, 1.0%; yeast extract, 0.3%; $(NH_4)_2SO_4$, 0.3%; NaCl, 0.2%; K_2HPO_4, 0.1% and $MgSO_4 \cdot 7H_2O$, 0.05% in tap water. pH was adjusted to 7.0. Cells were grown in 500 ml of medium in 2 liters flask with shaking for 24 hr at 28°C, harvested by centrifugation and washed with deionized water. Cell were, then, suspended in 0.05 M potassium phosphate buffer (pH 7.0) and treated with Kaijo Denki ultrasonic oscillator at 15kC/sec for 10 min. The cell debris were removed by centrifugation at 10,000 rpm for 30 min. Ammonium sulfate was added to the cell-free extract so as to give 30 to 80% saturation. The precipitated protein was collected by centrifugation at 10,000 rpm for 20 min, dissolved in 0.05 M potassium phosphate buffer (pH 7.0), and dialyzed against the same buffer of 0.01 M. This protein solution was used as the enzyme preparation.

The cultivation and the preparation of cell-free extract of *Escherichia coli* 2bT were the same as in *Proteus mirabilis*.

Enzyme assay. In this study, unless otherwise mentioned,

the phosphoryl transferring reaction was carried out with the standard reaction mixture containing 10 μmoles of NAD, 40 μmoles of phosphoryl donor, 20 μmoles of nicotinamide, 1 μmole of zinc sulfate, 300 μmoles of acetate buffer (pH 4.0) and the ammonium sulfate fraction (5 mg of protein) in a total volume of 1 ml. After incubation at 37°C with occasional stirring, the reaction was stopped by heating the mixture in boiling water for 3 min. Denatured protein was removed by centrifugation and the supernatant was subjected to quantitative analysis of substrates and products.

Analysis. Substrates and products in the reaction mixture were determined fluorometrically by a modified method of Carpenter and Kodicek.[54] The substances were separated by paper chromatography using a solvent system consisting of 10 parts of isobutyric acid and 6 parts of 0.5 N ammonium hydroxide.[55] Substance on the paper were detected with UV lamp. Each spot corresponding to nucleotides was cut off and extracted with 0.01 N hydrochloric acid for 15 to 20 hr at room temperature. To 1 ml of the extract, 0.2 ml of methyl ethyl ketone containing 2×10^{-4} M manganese sulfate and 0.6 ml of 3.5 N sodium hydroxide solution were added. After standing for 5 min at room temperature, 6.2 ml of 0.5 N hydrochloric acid was added and the mixture was immersed into boiling water for 5 min. Generated fluores-

cence was measured at 460 mμ using a fluorometer attachment of Hitachi spectrophotometer model EPU-II A. One tenth N sulfuric acid solution of quinine sulfate was used as the standard of fluorescence. Nucleotides in the extract were also determined by measuring the absorbancy at 260 mμ with a Hitachi spectrometer model EPU-II A or Hitachi Perkin-Elmer spectrophotometer model 139.

NADP could not be determined by usual spectrophotometric methods using enzyme such as isocitrate[56] or glucose 6-phosphate[57] dehydrogenase, because *p*-NPP and *p*-NP in the reaction mixture had a strong absorbancy at 340 mμ. In this work, therefore, the coenzyme activity was determined by the combined reactions of aconitase, isocitrate and glutamate dehydrogenases of dialyzed cell-free extracts of *Escherichia coli* 2bT with the reaction mixture containing 200 μmoles of sodium citrate, 400 μmoles of ammonium chloride, 10 μmoles of magnesium sulfate, 250 μmoles of Tris-HCl buffer (pH 7.4) and the dialyzed cell-free extract (20 mg of protein) in a total volume of 5 ml. After incubation for 2 to 4 hr at 37°C, the reaction was stopped by the addition of equivolume of ethanol. Glutamic acid formed was separated by circular paper chromatography using a solvent system consisting of 4 parts of n-butanol, 1 part of acetic acid and 1 part of water. The filter paper was sprayed with a 0.5%

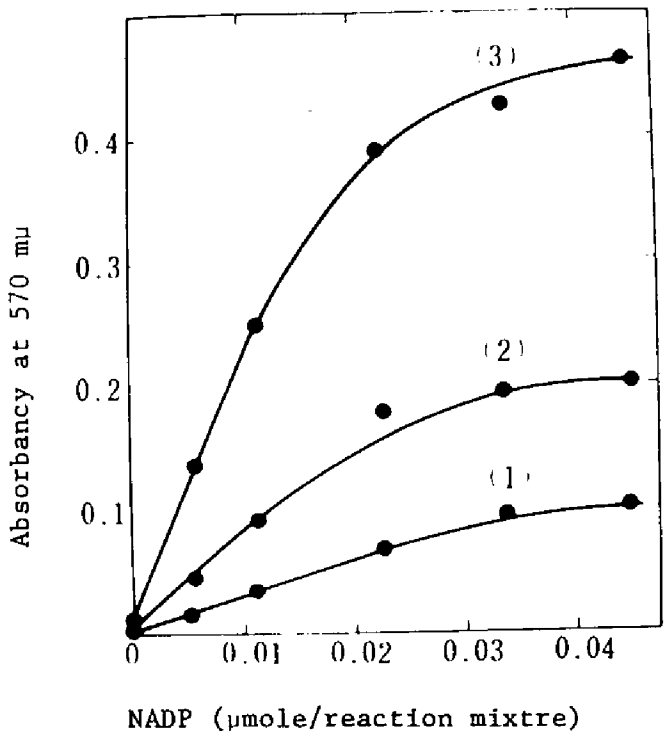


Fig. 1. Enzymic Determination of NADP.

(1) 1 hr incubation
(2) 2 hr incubation
(3) 4 hr incubation

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solution of ninhydrin in 75% ethanol, and after drying, kept at 50°C for 30 min. A deep purple spot developed was cut off and extracted with 5 ml of 75% ethanol. After 30 min, the absorbancy at 570 mμ was measured by a Hitachi photoelectric photometer model EPO-B. NADP below 0.025 μmole was proportional to glutamate formed as shown in Fig. 1. The activity of glutamate dehydrogenase varied with the enzyme preparation and, therefore,

NADP solutions of known concentration were incubated with the enzyme in each experiment. The enzyme preparation did not have any NADP synthesizing activity from NAD and *p*-NPP under these conditions.

Preparation of dehydrogenases. Isocitrate dehydrogenase was prepared from baker's yeast according to the method of Kornberg.[58] This preparation showed aconitase activity besides isocitrate dehydrogenase activity. Glucose 6-phosphate dehydrogenase of yeast was obtained from Sigma Chemical Co.

RESULTS

I. Formation of NADP from NAD and p-NPP by Proteus mirabilis

Time course of the reaction and detection of the products. When *p*-NPP was added in the reaction mixture described under Materials and Methods, an UV absorbing spot which also showed fluorescence after exposure to the mixed vapor of methyl ethyl ketone and ammonia[59] was developed below the spot of NAD as shown in Fig. 2. This spot was eluted with 5 ml of 0.01 N hydrochloric acid and determined fluorometrically. NADP was determined enzymatically using glutamate dehydrogenase. It was demonstrated that the only small portion (less than 30% yield) of the products determined as NADP fluorometrically was enzymatically active NADP. The results suggested that the product was a mixture

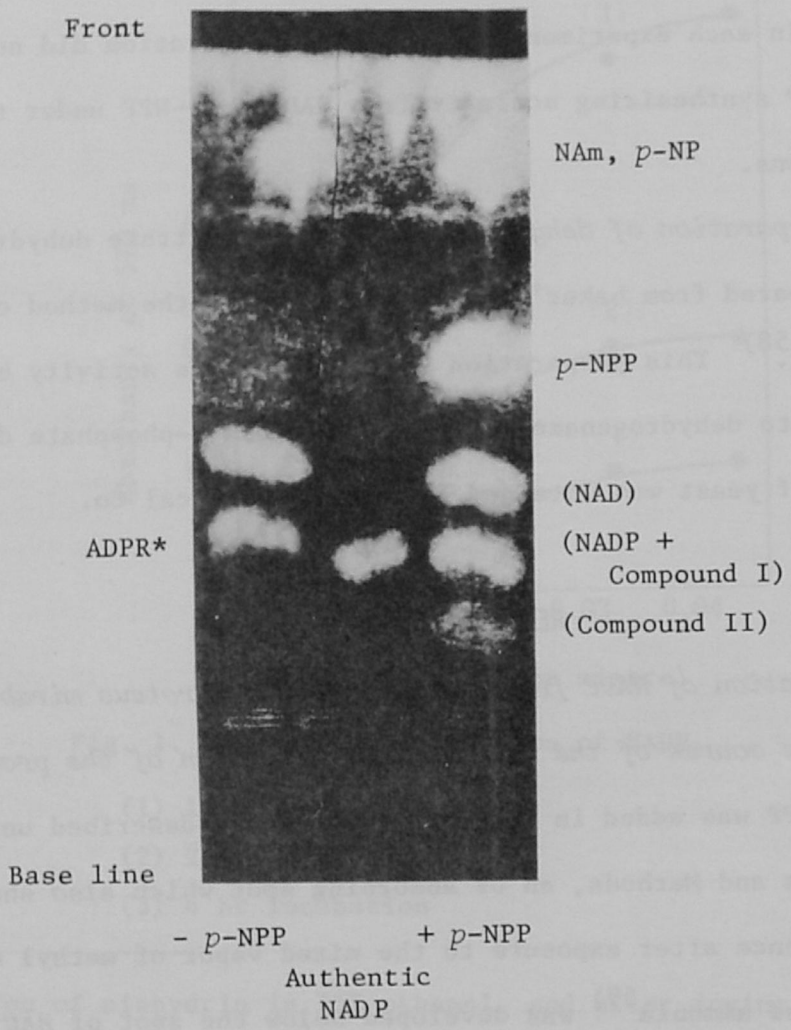

Fig. 2. Separation of Reaction Products by Paper Chromatography with Isobutyric Acid-Ammonia System.

Parentheses indicate the spot which showed fluorescence after methyl ethyl ketone treatment.

* ADPR was identified by comparing it with the degradation product of NAD by NADase from *Neurospora crassa*.[60]

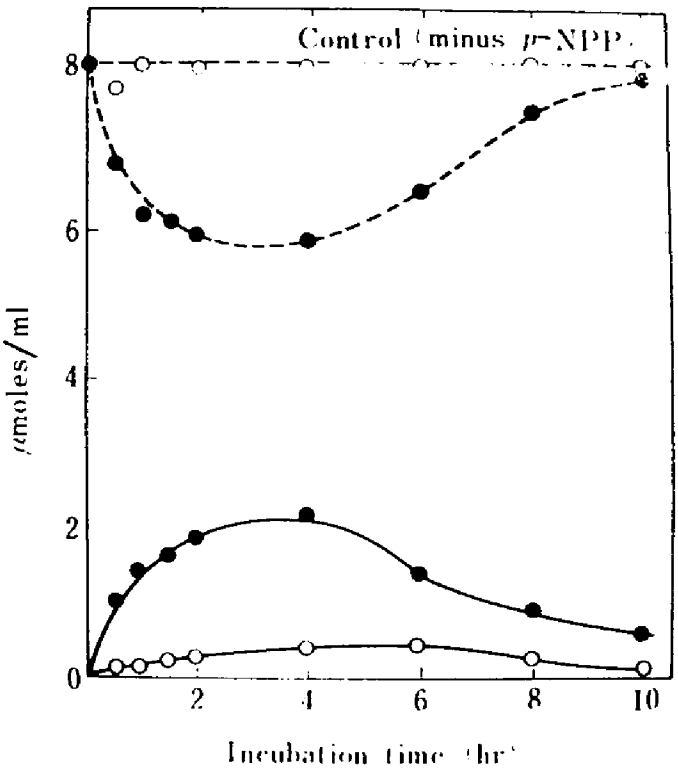

Fig. 3. Time Course of NAD Phosphorylation.

●——● NADP + Compound I (determined fluorometrically)

o——o NADP (determined enzymatically)

●- - -● NAD

o- - -o NAD (control; minus *p*-NPP)

The reaction mixture (5 ml) contained 50 μmoles of NAD, 200 μmoles of *p*-NPP, 100 μmoles of NAm, 5 μmoles of $ZnSO_4$, 1.5 mmoles of acetate buffer (pH 4.0) and the ammonium sulfate fraction* (25 mg protein). Incubation was carried out at 37°C with occasional stirring. ADPR (1.2 – 1.5 μmoles per ml) was detected at 0 hr with or without *p*-NPP but was not shown in the figure.

* See Material and Methods.

of NADP and an unidentified substance possessing no coenzyme activity for glutamate dehydrogenase. The unidentified substance was expressed as Compound I in this section. Below Compound I on the paper chromatogram there was another spot (Compound II) which showed fluorescence by the methyl ethyl ketone treatment. On the other hand, in the absence of *p*-NPP, no NADP and Compounds I and II was synthesized. Figure 3 shows time course of the reaction in the presence or absence (control) of *p*-NPP. The formation of NADP and Compound I was observed only in the presence of *p*-NPP and reached a maximum after 2 to 4 hr of the incubation, then decreased gradually. NADP might be produced by the action of phosphatase as a degradation product. This degradative reaction was inhibited by the addition of *p*-NPP during the incubation. This phenomenon will be mentioned later.

Phosphoryl donor specificity. Phosphoryl transferring reaction was investigated using NAD and various phosphoryl donors. As shown in Table I, *p*-NPP was the most effective for both NADP and Compound I formation. When purine nucleotides were used as phosphoryl donor, 5'-IMP is the most effective. 2'(3')-AMP was more effective than 5'-isomer, whereas in GMP no difference between 5'- and 3'-isomers was observed. On the other hand in pyrimidine nucleotides 3'-isomers were effective, whereas 5'-isomers have almost no activity as phosphoryl donor. From the

TABLE I. EFFECT OF PHOSPHORYL DONOR ON PHOSPHORYLATION OF NAD

Phosphoryl donor	NADP + Compound I formed µmoles/ml	NADP formed µmole/ml
5'-AMP	0.24	trace
2'(3')-AMP	0.52	trace
5'-GMP	0.26	0.01
2'(3')-GMP	0.28	0.01
5'-IMP	0.57	0.03
5'-CMP	0.05	trace
2'(3')-CMP	0.45	0.01
5'-UMP	0.05	trace
2'(3')-UMP	0.24	0.01
ATP	0.05	0
p-NPP	2.10	0.22
None	0.02	0

The reaction mixture (1.25 ml) contined 12.5 µmoles of NAD, 50 µmoles of phosphoryl donor, 1.25 µmoles of zinc sulfate and the ammonium sulfate fraction (6.25 mg protein). Incubation was carried out at 37°C for 4 hr.

fact that ATP did not serve as phosphoryl donor at all it might be concluded that this transferring reaction was apparently different from that catalyzed by NAD kinase.

Effect of pH. Effect of pH on phosphoryl transferring reaction between *p*-NPP and NAD was studied using the ammonium

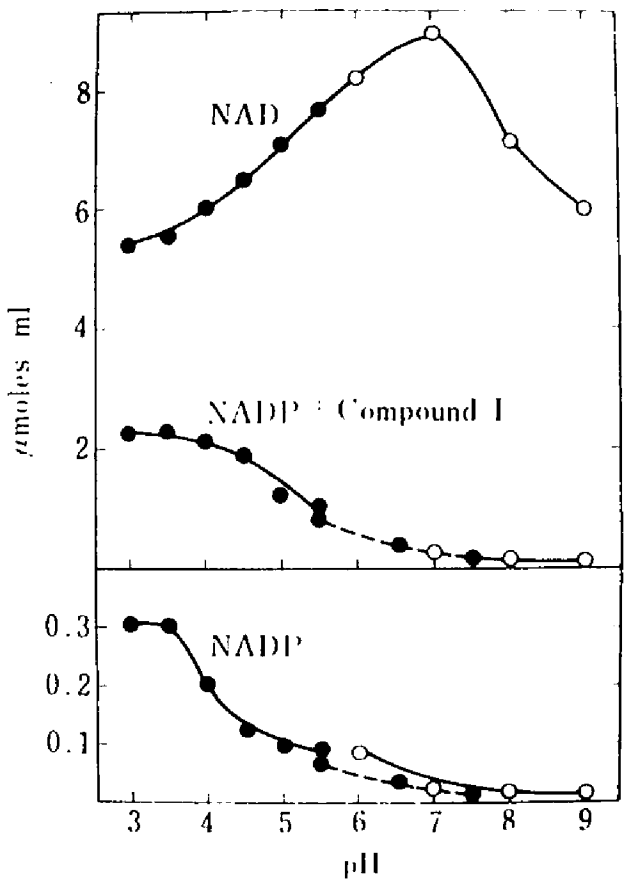

Fig. 4. Effect of pH on Phosphorylation of NAD.

Each buffer was added to a final concentration of 0.3 M. Incubation was carried out for 4 hr at 37°C under the standard conditions.

●——● Acetate buffer

●----● Tris-HCl buffer

○——○ Tris-Maleate buffer

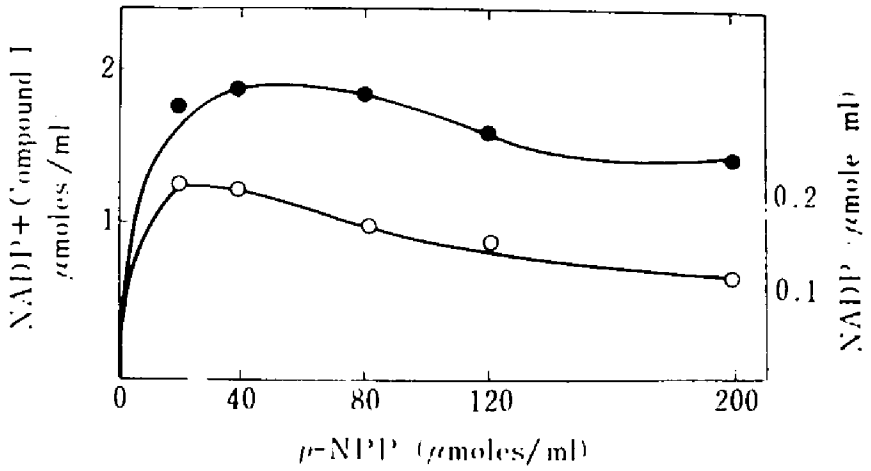

Fig. 5. Effect of *p*-NPP Concentration on Phosphorylation of NAD.

Incubation was carried out at 37°C for 2 hr.

●——● NADP + Compound I ○——○ NADP

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sulfate fraction (see Materials and Methods). As shown in Fig. 4, the optimal pH for both NADP and Compound I formation was below 4.0. In alkaline pH the transfer reaction decreased greatly. This may be caused by nonenzymatic degradation of the nicotinamide riboside bond. The kinds of the buffer tested did not affect on the reaction.

Effect of p-NPP concentration. As shown in Fig. 5, optimal concentration of *p*-NPP was 40 μmoles per ml of the reaction mixture for the formation of Compound I and 20 μmoles for that of NADP. When more *p*-NPP was added, the reaction was slightly inhibited.

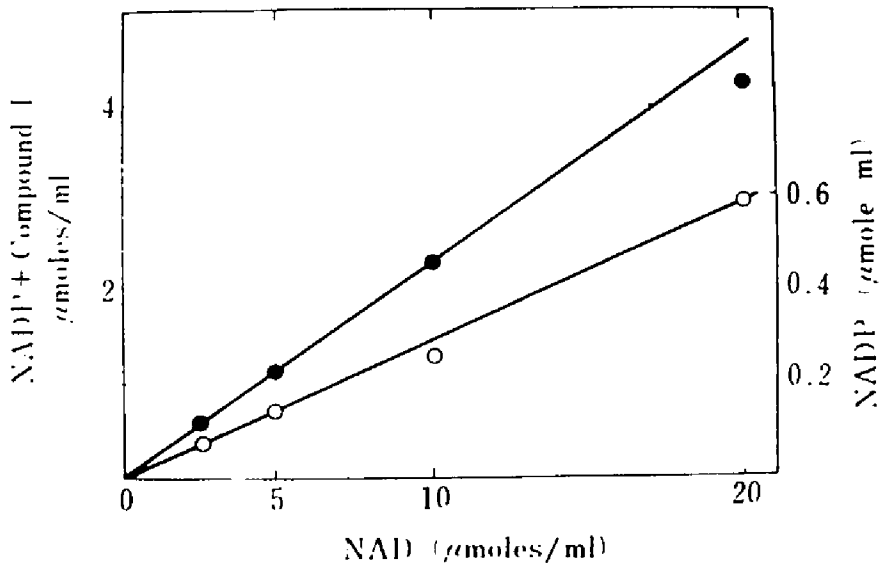

Fig. 6. Effect of NAD Concentration of Phosphorylation of NAD.

●——● NADP + Compound I O——O NADP

The reaction mixture (3 ml) contained various amounts of NAD as shown in the figure, 120 μmoles of *p*-NPP, 450 μmoles of acetate buffer (pH 4.0) and the ammonium sulfate fraction (10 mg protein). Incubation was carried out at 37°C for 6 hr.

Effect of NAD concentration. Under the conditions tested, the formation of Compound I and NADP was approximately proportional to NAD concentration as shown in Fig. 6.

Effect of enzyme concentration and incubation time. Effect of enzyme concentration on NAD phosphorylation was investigated with the ammonium sulfate fraction of *P. mirabilis*. The results are shown in Fig. 7. When incubated for 2 hr, the for-

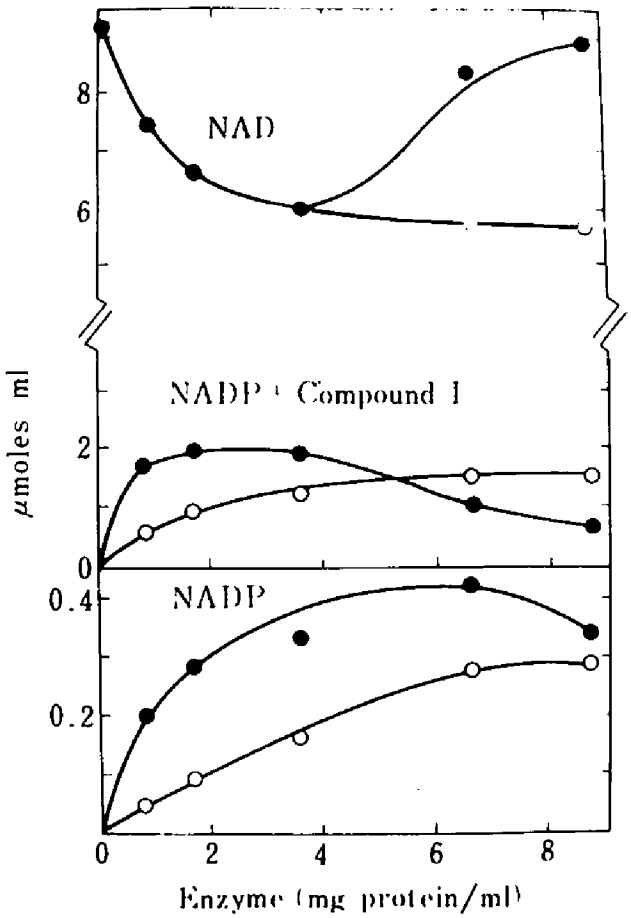

Fig. 7. Effect of Enzyme Concentration on Phosphorylation of NAD.

○——○ 2 hr incubation ●——● 6 hr incubation

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mation of NADP and Compound I was proportional to the enzyme concentration. On the other hand, when incubated for 6 hr, the phosphorylation was maximal in the presence of about 3 mg protein of the enzyme per ml of mixture, and with the higher con-

centration of enzyme the secondary degradation of the products and the regeneration of NAD took place.

II. Isolation of NADP

Separation of the products by column chromatography.

For the isolation of the reaction products, following reaction system was employed; 600 μmoles of NAD, 2.4 mmoles of *p*-NPP, 45 mmoles of acetate buffer (pH 4.0), 60 μmoles of zinc sulfate and the ammonium sulfate fraction of *P. mirabilis* (316 mg of protein) in a total volume of 60 ml. The reaction mixture was incubated at 37°C for 8 hr with occasional stirring. In order to inhibit the secondary degradation of the products, 1.2 mmoles of *p*-NPP were added after 2, 4 and 6 hr incubation. Aliquots of the reaction mixture were taken out hourly and subjected to quantitative analysis of the products. Time course of the reaction is shown in Fig. 8. After 8 hr incubation, reaction was stopped by heating the mixture in boiling water for 5 min, and denatured protein was removed by centrifugation. The supernatant was adjusted to pH 6.8 with ammonium hydroxide and placed on a Dowex 1 x 2 (in formate form) column. After washing with water, the reaction products were eluted with increasing concentration of formate buffer (pH 3.4). The optical density of eluates, collected in 10 ml fractions, were read at 260 mμ with the spectro-

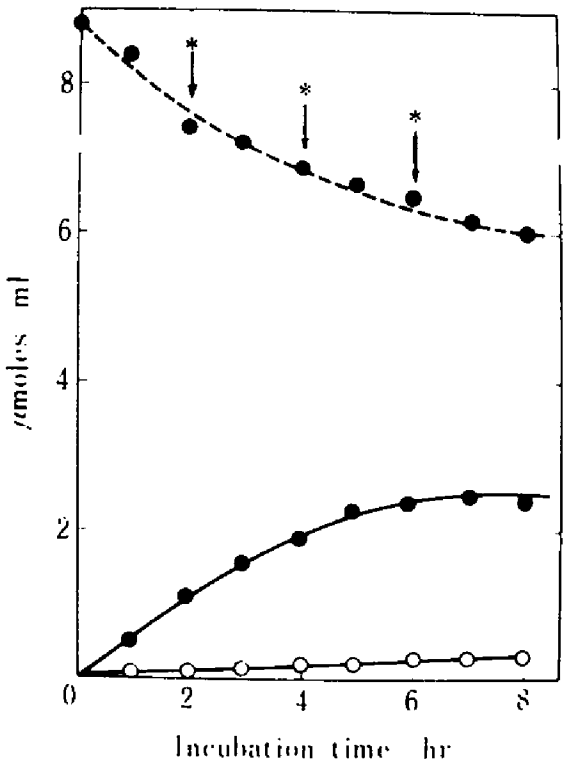

Fig. 8. Time Course of Reaction and Effect of *p*-NPP Addition.

* *p*-NPP addition
●——● NADP + Compound I
O——O NADP
●- - -● NAD

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photometer to follow the appearance of nucleotides. The elution pattern is presented in Fig. 9. The amounts of *p*-NPP and *p*-NP were followed by measuring absorbancy at 290 mμ and 320 mμ respectively; *p*-NPP was eluted after ADPR.

NADP and Compound I fractions were collected, adsorbed by

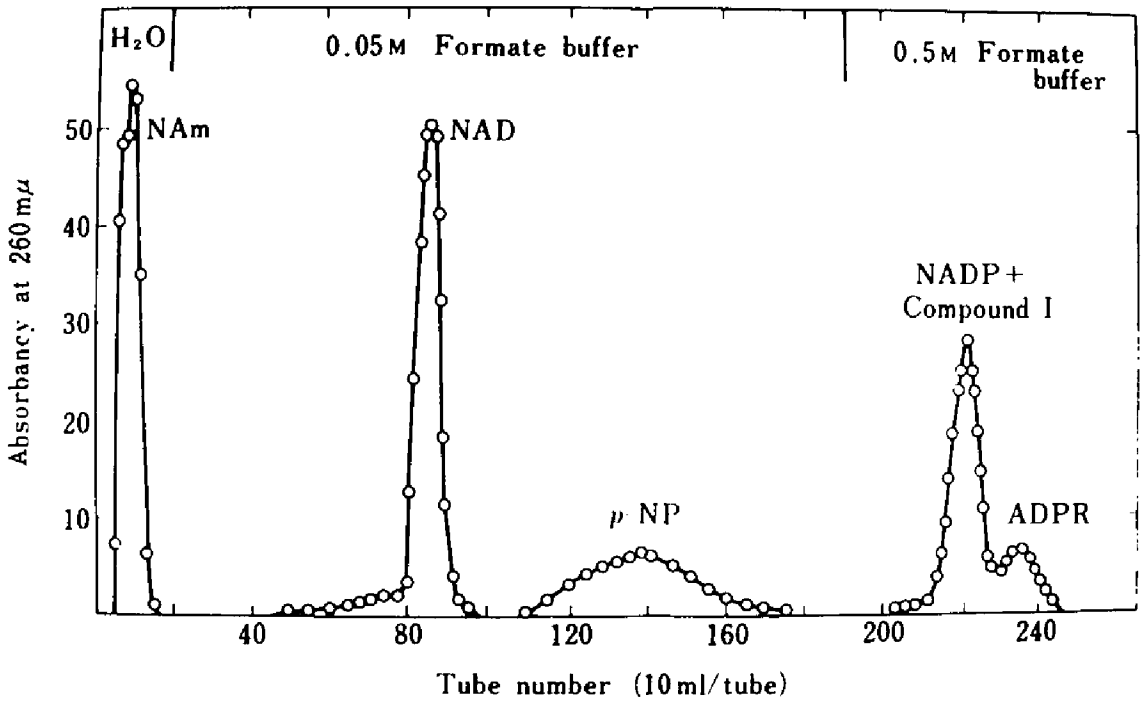

Fig. 9. Separation of Reaction Products by Column Chromatography on Dowex 1.

Column: Dowex 1 x 2, formate form, 200-400 mesh, 1.75 cm^2 x 25 cm.

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500 mg of active charcoal and eluted with 200 ml of a mixture of pyridine, ethanol and water (10 : 45 : 45). The extract was concentrated by a rotary evaporator. After reprecipitation from cold acetone, 76 mg of white powder was obtained.

NADP activity of the isolate. The isolated powder was dissolved in water and tested for NADP activity using aconitase and NADP-linked isocitrate dehydrogenase preparations obtained

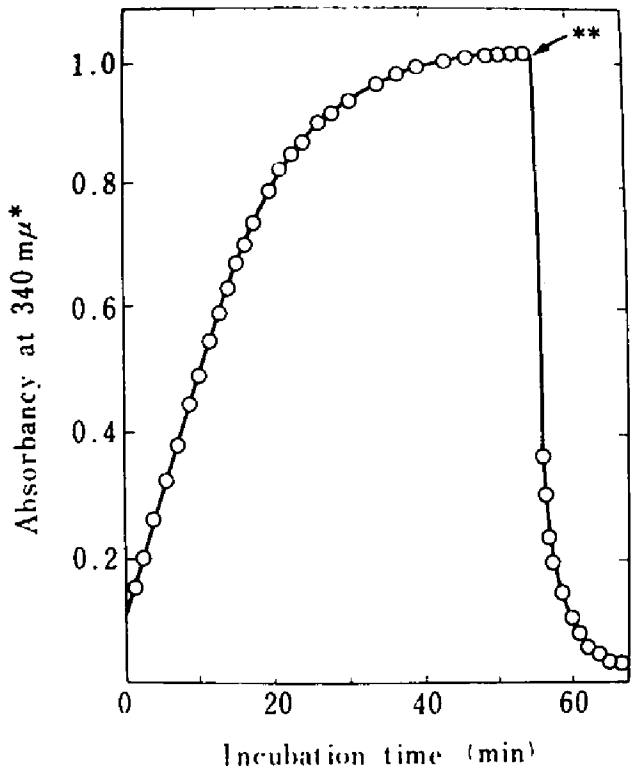

Fig. 10. Reduction of NADP by Isocitrate Dehydrogenase.

The reaction mixture (3.5 ml) contained 2.5 μmoles of citrate, 167 μmoles of phosphate buffer (pH 7.0), 17 μmoles of $MgCl_2$, 4 mg of the isolate (a mixture of NADP and Compound I) and isocitrate dehydrogenase (2.5 mg protein). Incubation was carried out at room temperature.

* Increase of absorbancy at 340 mμ was measured with a cell of 1.0 cm light path.

** 2N hydrochloric acid was added.

from baker's yeast. Results are shown in Fig. 10. Remarkable increase in optical density at 340 mμ caused by the enzymic reduction of NADP was observed. When the reduction was completed, pH of the reaction mixture was adjusted to 2.0 with hydrochloric acid. The reduced coenzyme was decomposed and a striking decrease of the absorbancy was observed. The isolated preparation was also reduced by glucose 6-phosphate dehydrogenase.

DISCUSSION

It is well known that NADP is synthesized by NAD kinase and that phosphoryl donor of the reaction is strictly limited to ATP and other nucleotides can not replace ATP.

On the other hand, Morton[61] and Brawerman and Chargaff[62,63] studied the enzymatic synthesis of nucleoside monophosphate by phosphotransferases other than nucleoside kinase. They observed the synthesis of nucleotides from nucleosides by phosphoryl group transfer of organic phosphates, such as phenylphosphate and nucleoside monophosphates, and suggested that this phosphorylating reaction might be one of the general pathway for biosynthesis of nucleotides.

The present report suggests that in bacterial system, such as *Proteus mirabilis*, NADP could be synthesized by phosphotransferase other than NAD kinase. When NAD and *p*-NPP were incubated

with the enzyme preparation of *Proteus mirabilis* (IFO 3849), NADP was formed in the reaction mixture. Furthermore, it was shown that ATP was unable to serve as the phosphoryl donor for NADP formation, whereas nucleoside monophosphates were effective. This fact indicates that phosphotransferase or phosphatase participates in the NAD phosphorylating reaction. In case of pyrimidine nucleotides 2'(3')-isomers were preferentially utilized for NADP synthesis. This might suggest that the phosphoryl group at 2'(3') position of nucleoside monophosphates was easily transferred to the 2'(3') position of adenosine moiety of NAD. Mitsugi *et al.*[51,52] reported that the enzyme preparation of *P. mirabilis* transferred the phosphoryl group of *p*-NPP to 2' or 3' position of nucleosides and that this organism phosphorylated nucleoside-5'-monophosphate to form nucleoside 2'(or 3'),5'-diphosphate. It would not be discussed here whether or not this NAD phosphorylating reaction may be catalyzed by the same enzyme.

Optimal pH for the NADP formation by phosphoryl transfer from *p*-NPP or nucleoside monophosphate was below 4.0, but that for NAD kinase was around 7.0. Moreover, this phosphotransferring enzyme reaction proceeded even in the absence of magnesium ion. These facts provided a strong evidence for the participation of the phosphotransferase other than NAD kinase in NADP synthesis.

It was observed that besides NADP, Compound I was formed in

the reaction mixture when the phosphotransferase preparation was incubated with NAD and *p*-NPP. The yield of NADP formed was less than 30% of the total phosphorylation products.

Structure and chemical properties of the Compound I will be discussed in the next section.

SUMMARY

NADP was enzymatically synthesized from NAD and *p*-NPP or nucleoside monophosphate with the enzyme preparation of *Proteus mirabilis* (IFO 3849). In this phosphotransferring reaction, ATP did not serve as phosphoryl donor.

In addition to NADP, an unidentified substance (Compound I) showing fluorescence with methyl ethyl ketone and having no coenzyme activity to glutamate dehydrogenase was synthesized. The yield of NADP was usually below 30 % of Compound I.

NADP was isolated from the reaction mixture and its coenzyme activity to some dehydrogenases was demonstrated.

Section 2. Chemical Structure of an NADP Analog

INTRODUCTION

In the previous section, the author showed that NADP was formed from NAD and *p*-NPP by the enzyme preparation of *Proteus mirabilis* (IFO 3849). Furthermore, in addition to NADP, a new derivative of NAD (Compound I), which showed the same fluorescence property as pyridine coenzymes by the treatment with methyl ethyl ketone, was synthesized.

This section deals with the isolation and identification of Compound I, Compound III which was the degradation product of Compound I by nucleotide pyrophosphatase of snake venom, and Compound IV which was formed by phosphorylation of 5'-NMN.

MATERIALS AND METHODS

Cultivation of Proteus mirabilis and preparation of the enzyme solution. Cultivation of *Proteus mirabilis* (IFO 3849) and the preparation of ammonium sulfate fraction of the enzyme solution were carried out as described in the previous section.

Separation of pyridine coenzymes. The following solvent systems were employed for the separation of pyridine coenzymes and the related compounds by paper chromatography: (a) isobutyric acid : 0.5 N ammonium hydroxide = 10 : 6,[55] (b) 95% ethanol :

1 M ammonium acetate = 7 : 3,[64] (c) saturated ammonium sulfate solution : 0.1 M sodium acetate : isopropanol = 79 : 19 ; 2.[65] The substances on paper were detected with an UV lamp and the methyl ethyl ketone method as mentioned in the previous section. High voltage paper electrophoresis was carried out with 0.2 M acetate buffer (pH 3.5) or 0.1 M borate buffer (pH 9.4); n-hexane was used as coolant. The apparatus was made by the Shiraimatsu Mechanical Industry Co., Ltd.

Analysis. Nicotinamide ribose linkage in the sample solutions or in the chromatographic fractions was assayed fluorometrically according to the methyl ethyl ketone method as mentioned in the previous section. Total phosphate was assayed by determining the inorganic phosphate after digestion of the sample with sulfuric acid by the methods of Fiske-SubbaRow[66] and Takahashi.[67] Phosphate liberated by phosphomonoesterases was also determined by these methods. Nicotinamide was assayed by modifying the cyanogen bromide method of Lamb[68] as follows: sample solution (0.5 ml) containing less than 1.0 μmole of nicotinamide was mixed with 0.5 ml of 0.2 N sodium hydroxide and after boiling for 5 min, 4 ml of water, 0.5 ml of 4% ethanol solution of aniline and 3 ml of 2.5% cyanogen bromide were added successively. Absorbancy at 420 mμ was measured after 4 to 5 min, when the absorbancy reached a maximum. Nicotinamide

concentration was calculated from a calibration curve prepared with an authentic nicotinamide solution treated with sodium hydroxide. Ribose was assayed by orcinol reaction.[69] Protein was determined according to the method of Lowry,[70] and in the case of purified phosphomonoesterases, according to the method of Warburg and Christian.[71]

Enzymes. NADP specific isocitrate dehydrogenase was prepared from baker's yeast according to the method of Kornberg.[58] 3'-Nucleotidase was prepared from germinating wheat[72] and the culture broth of *Bacillus subtilis* (IAM 1193)[73] was also used as the nucleotidase in some experiments. 5'-Nucleotidase was obtained from bull semen according to the method of Heppel *et al.*[74] Purified nonspecific phosphomonoesterase prepared from human prostatic gland by the method of Schmidt[75] and partially purified 3'-nucleotidase from culture broth of *B. subtilis* were kindly supplied by Dr. Y. Sugino. NADase was obtained from *Neurospora crassa* (IFO 6068) grown in a zinc-deficient medium according to the method of Kaplan *et al.*[60] Nucleotide pyrophosphatase was purified from lyophilized powder of Mamushi (*Agkistrodon halys blomohoffii*) venom by modifing the method of Suzuki *et al.*[76] using column chromatography on DEAE-cellulose and CM-cellulose. This pyrophosphatase preparation was found to catalyze the degradation of both NAD and calcium bis-*p*-nitrophenyl

phosphate, but not of 5'-AMP. Yeast alcohol dehydrogenase was obtained from C. F. Boehringer and Soehne GmbH.

RESULTS

I. Isolation of Compound I

In order to isolate the new derivative of NAD (Compound I) the incubation was carried out for 8 hr at 37°C using the reaction mixture contained 1 mmole of NAD, 2 mmoles of nicotinamide, 100 μmoles of zinc sulfate and 4 mmoles of *p*-NPP. Two mmoles each of *p*-NPP were added after 2, 4 and 6 hr of incubation. The reaction products were separated by column chromatography on Dowex 1 x 2 in formate form. Stepwise elution was carried out with formate buffer (pH 3.5); NADP and Compound I were eluted with 0.5 M formate buffer. The fractions were collected and 3 volumes of cold acetone were added. The precipitates were collected by centrifugation, then washed twice with cold acetone and cold ethyl ether (yield of white powder, 100 mg). The white powder was dissolved in water and NADP presented in the preparation was reduced enzymatically. The reaction mixture consisted of 140 mg of white powder (mixture of NADP and Compound I), 150 μmoles of sodium citrate, 750 μmoles of magnesium sulfate, 7.5 mmoles of potassium phosphate buffer (pH 7.0) and the isocitrate dehydrogenase preparation which also exhibited aconitase activity

(75 mg as protein) in a total volume of 105 ml. The reaction was followed by measuring the increase in absorbancy at 340 mμ. When the reduction reached maximum, 2 N hydrochloric acid was added to degrade $NADPH_2$. After denatured protein was removed by centrifugation, pH of the supernatant was adjusted to 7.0 with ammonium hydroxide, and the precipitate formed was centrifuged off. The clear supernatant was again applied to the column of Dowex 1 x 2 in formate form and Compound I was eluted by 0.5 N formate buffer. To this fraction was added 5 volumes of cold acetone and resulting precipitate was collected by centrifugation. Finally, the precipitate was washed with cold acetone and ethyl ether. The yield of white powder was about 100 mg.

II. Chemical properties of Compound I

Spectral analysis. Compound I obtained above was dissolved in water and the spectral properties were investigated. As shown in Fig. 1, this compound has an absorption maximum at 259 mμ. By addition of potassium cyanide, the absorption at 259 mμ was reduced and a new peak at 327 mμ appeared. The reduced Compound I has an absorption maximum at 340 mμ. All of these spectral characteristics in the UV range were very similar to those of the other pyridine coenzymes.

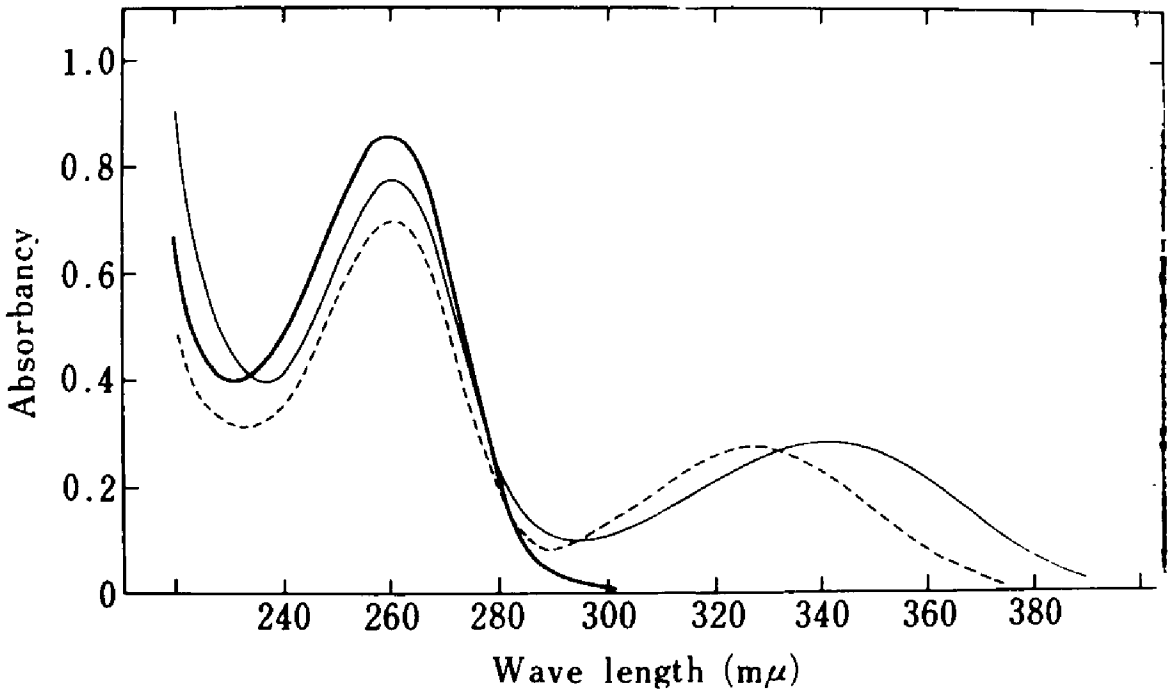

Fig. 1. Ultraviolet Absorption Spectra of Compound I.

——— Oxidized form ——— Reduced form

------ Cyanide complex

The concentration of Compound I was 4.7×10^{-5}M. Compound I was reduced by sodium hydrosulfite.[77] The cyanide complex was formed in 0.1 M potassium cyanide solution.[78]

For the determination of the concentration of Compound I, several methods were examined using NADP as a standard specimen. At first, the concentration of Compound I was determined by measuring the amount of nicotinamide ribose moiety fluorometrically. The values calculated from absorbancy at 260 mμ, 340 mμ of the

TABLE I. DETERMINATION OF COMPOUND I

Fluoro-metry	Calculated from*		
	Absorb. at 260 mμ of oxidized form	Absorb. at 340 mμ of reduced form	Absorb. at 327 mμ of cyanide complex
1.00	1.02	1.00	0.97

Figures show the ratios of calculated concentration against that determined fluorometrically.

* Estimated based on molar extinction coefficients of 18.0 x 10^6 cm^2, 6.2 x 10^6 cm^2 and 5.9 x 10^6 cm^2, for oxidized, reduced forms and cyanide complex, respectively.

reduced form, and 327 mμ of the cyanide complex, were identical with that obtained by fluorometry (Table I).

Electrophoretic behavior of Compound I. As shown in Table II, Compound I showed the same mobility as NADP in both acidic and alkaline conditions. This fact suggested that the compound possessed the same electric charge as NADP.

Behaviors in paper chromatography. As shown in Table II, Compound I has the similar *Rf* values as that of NADP but apparently has different *Rf* values from those of the other pyridine derivatives. In solvent (c) *Rf* values of Compound I and NADP were slightly different from each other.

TABLE II. BEHAVIORS OF COMPOUNDS I, III AND IV IN PAPER CHROMATO-

Experiment No.	Compound	*Rf* values	
		Isobutyric acid-ammonia	Am-acetate ethanol
1	NAD	(0.34)	(0.22)
	NADP	(0.25)	(0.10)
	Compound I	(0.24)	(0.10)
	Nicotinamide	0.98	0.87
	Adenosine	0.94	0.66
	5'-AMP	0.49	0.42
	5'-NMN	(0.45)	(0.46)
	Degradation products of NADP	0.29 (ADP)	0.27
		(0.43) (NMN)	(0.44)
		(0.89) (NAm-R)	(0.69)
		0.99 (NAm)	0.88
	Degradation products of Compound I	(0.26) (Comp. III)	(0.21)
		0.94 (Ad-R)	0.67
		0.99 (NAm)	0.86
2	5'-NMN	(0.50)	(0.43)
	NAm-R**	(0.75)	(0.77)
	Compound III	(0.34)	(0.24)
	Compound IV	(0.33)	(0.24)

Parentheses indicate the spot which showed fluorescence by methyl

* The reaction mixture of the following system was used to prepare acetate buffer (pH 5.5), 150 μmoles; prostatic phosphatase, 0.48

** Plus and minus indicate migration distance in centimeter to the

GRAPHY AND HIGH VOLTAGE PAPER ELECTROPHORESIS

	Mobilities*	
Am-sulfate-Na-acetate-isopropanol	0.2 M acetate buffer, pH 3.5 3KV, 30mA, 60min	0.1 M borate buffer, pH 9.4 3KV, 30mA, 40min
(0.34)	(+ 4.0)	(+ 9.5)
(0.48)	(+ 13.0)	(+ 11.0)
(0.41)	(+ 13.0)	(+ 11.0)
0.40		
0.18	- 2.5	+ 3.5
0.34	+ 5.5	+ 11.0
(0.80)		

	3KV, 12mA, 60min	3KV, 20mA, 30min
(0.77)	(- 0.5)	(+ 11.0)
(0.74)	—	(- 1.5)
(0.82)	(+ 13.0)	(+ 15.0)
(0.82)	(+ 13.0)	(+ 15.0)

ethyl ketone treatment.

nicotinamide riboside: 5'-NMN, 0.14 μmole; $MgSO_4$, 10 μmoles; mg protein; total volume, 1.0 ml; incubation at 37°C for 6 hr. anode and cathode, respectively.

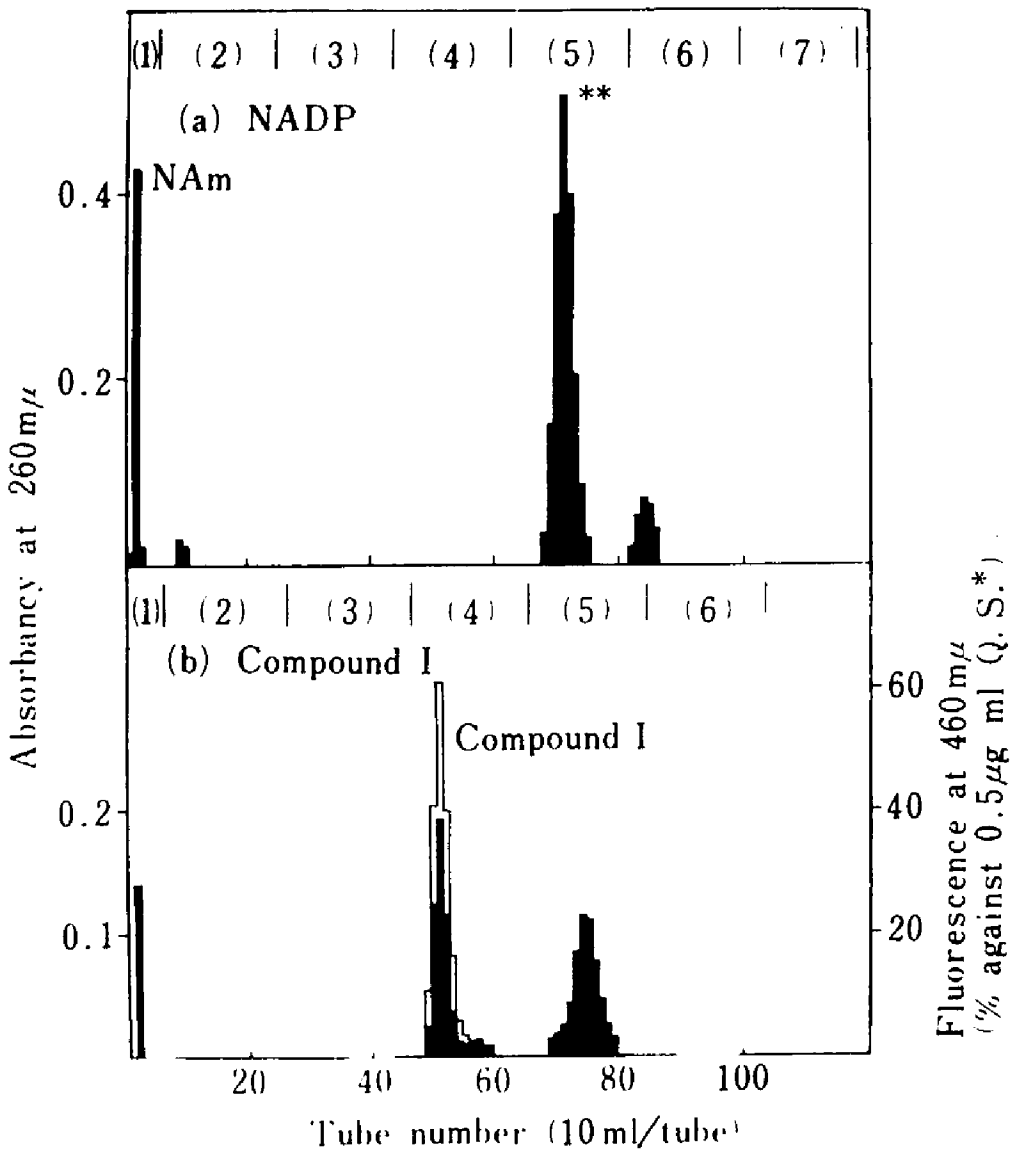

Fig. 2. Degradation of NADP and Compound I by NADase.

Absorbancy Fluorescence

The reaction mixture (2 ml) contained 4.87 μmoles of NADP or 2.98 μmoles of Compound I, 500 μmoles of phosphate buffer (pH 7.0), and NADase (0.62 mg protein). Incubation was carried

out at 37°C for 1.5 hr. 1.2 or 1.1 ml of the reaction mixture was applied to the column of Dowex 1 x 2 (formate form, 0.79 cm^2 x 12.5 cm).

(1) Water
(2) 0.05 M Formate buffer
(3) 0.2 M Formate buffer
(4) 0.5 M Formate buffer
(5) 1.0 M Formate buffer
(6) 2.0 M Formate buffer
(7) 4.0 M Formate buffer

* 0.1 N sulfuric acid solution of quinine sulfate.

** This fraction was supposed to be ADPR-phosphate.

III. Enzymic degradation of Compound I and isolation of the products

Degradation by NADase. NADP was hydrolyzed completely after 90 min incubation by NADase, whereas Compound I was degraded only 58%. The lower activity of NADase on Compound I might be due to the difference in chemical structure of the compound. The elution pattern of the reaction mixture from the column is shown in Fig. 2.

Degradation by snake venom. It is well known that the snake venom usually contains phosphodiesterase[79] (nucleotide pyrophosphatase[76]) and 5'-nucleotidase[80,81] which can not degrade 5'-phosphoryl group when another phosphoryl group is bound to the other hydroxyl group of the ribose moiety of nucleotides.[82]

The degradation products of NADP and Compound I were sepa-

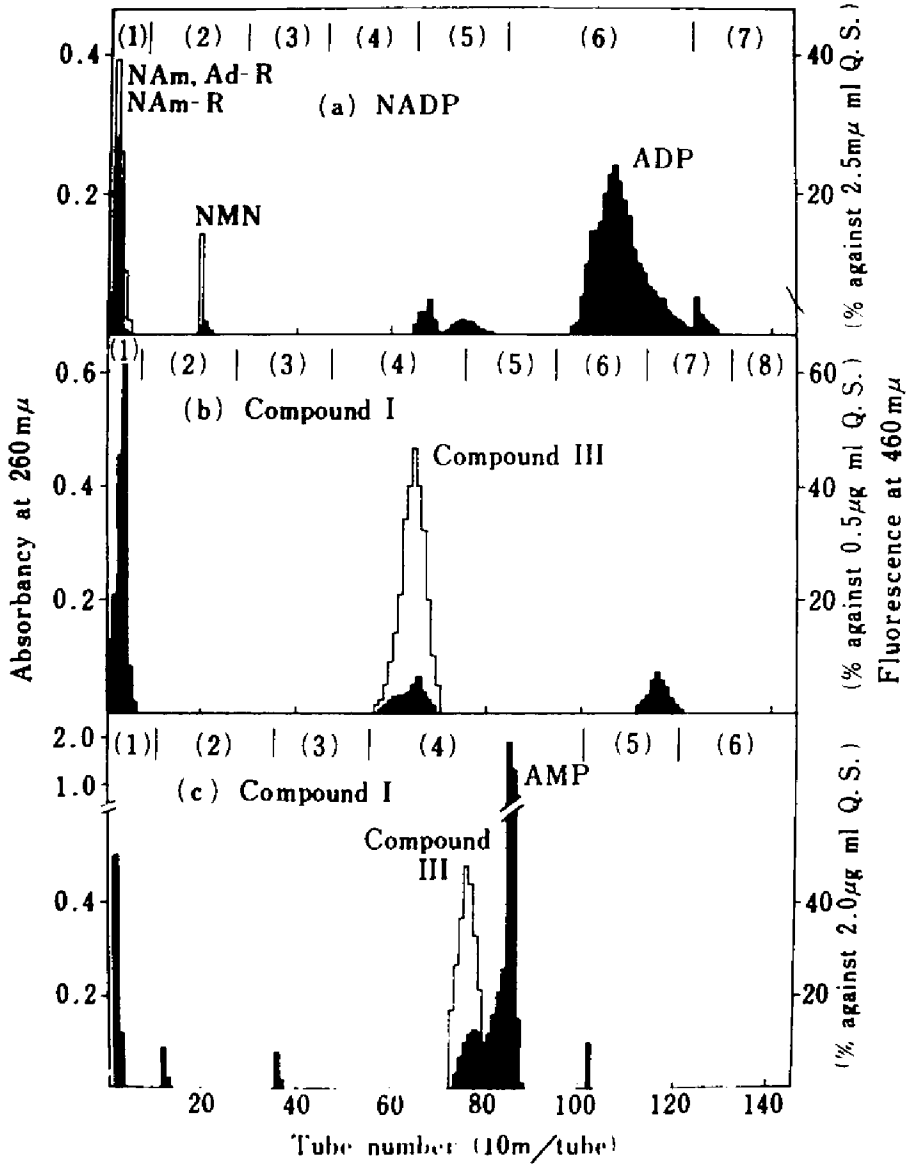

Fig. 3. Degradation of NADP and Compound I by Snake Venom and Purified Nucleotide Pyrophosphatase.

Absorbancy Fluorescence

(a)(b) The reaction mixture (2 ml) contained 1.98 μmoles of NADP or 2.38 μmoles of Compound I, 250 μmoles of Tris-HCl buffer (pH 7.2), and 2.5 mg of lyophylized powder of snake vonom. Incubation was carried out at 37°C for 2 hr. The supernatant solution was applied to a column of Dowex 1 x 2 (formate form, 0.78 cm^2 x 12.5 cm).

(c) The reaction mixture (2.4 ml) contained 4.5 μmoles of Compound I, 300 μmoles of Tris-HCl buffer (pH 7.0), and purified nucleotide pyrophosphatase (1.2 mg protein). Incubation was carried out at 37°C for 3 hr. The supernatant solution was applied to a column of Dowex 1 x 2 (formate form, 0.79 cm^2 x 14.5 cm).

(1) Water	(5) 0.2 M Formate buffer
(2) 0.01 M formic acid	(6) 0.5 M Formate buffer
(3) 0.02 M Formate buffer	(7) 1.0 M Formate buffer
(4) 0.05 M Formate buffer	(8) 2.0 M Formate buffer

* 0.1 N sulfuric acid solution of quinine sulfate.

Nicotinamide, nicotinamide riboside, NMN, adenosine and AMP were identified from the eluted position of the fraction. Furthermore, each fraction was adsorbed to charcoal, eluted with a pyridine-ethanol-water mixture, concentrated and developed by paper chromatography. *Rf* value of each substance was compared with that of authentic specimen.

ADP was identified by the determination of total phosphate and UV absorption at 260 mμ.

rated by paper chromatography and column chromatography. Table II and Fig. 3 show the *Rf* values of the degradation products and the elution pattern obtained by column chromatography, respectively. By the action of venom enzymes, NADP was degraded to nicotinamide, NMN, nicotinamide riboside and adenosine diphosphate (adenosine 2',5'-diphosphate).[83] On the other hand, Compound I was degraded to adenosine, nicotinamide and an unidentified compound (designated as Compound III) which showed fluorescence by exposure to methyl ethyl ketone-ammonia vapor. The *Rf* value of the Compound III was different from that of nicotinamide riboside and NMN. In column chromatography, the Compound III was eluted with 0.05 M buffer and detected fluorometrically. This fraction was not obtained from the digested products of NADP. The fact that Compound III was not formed from NADP indicated that the chemical structure of Compound I apparently differs from that of NADP.

Isolation of Compound III. Compound III eluted from the column with 0.05 M buffer was adsorbed on active charcoal, extracted with a pyridine-ethanol-water (10 : 45 ; 45) mixture and then concentrated under reduced pressure. The residue was dissolved in a small amount of water and 5 volumes of cold acetone was added. The resulting precipitate was collected by centrifugation and washed with acetone and ethyl ether. For

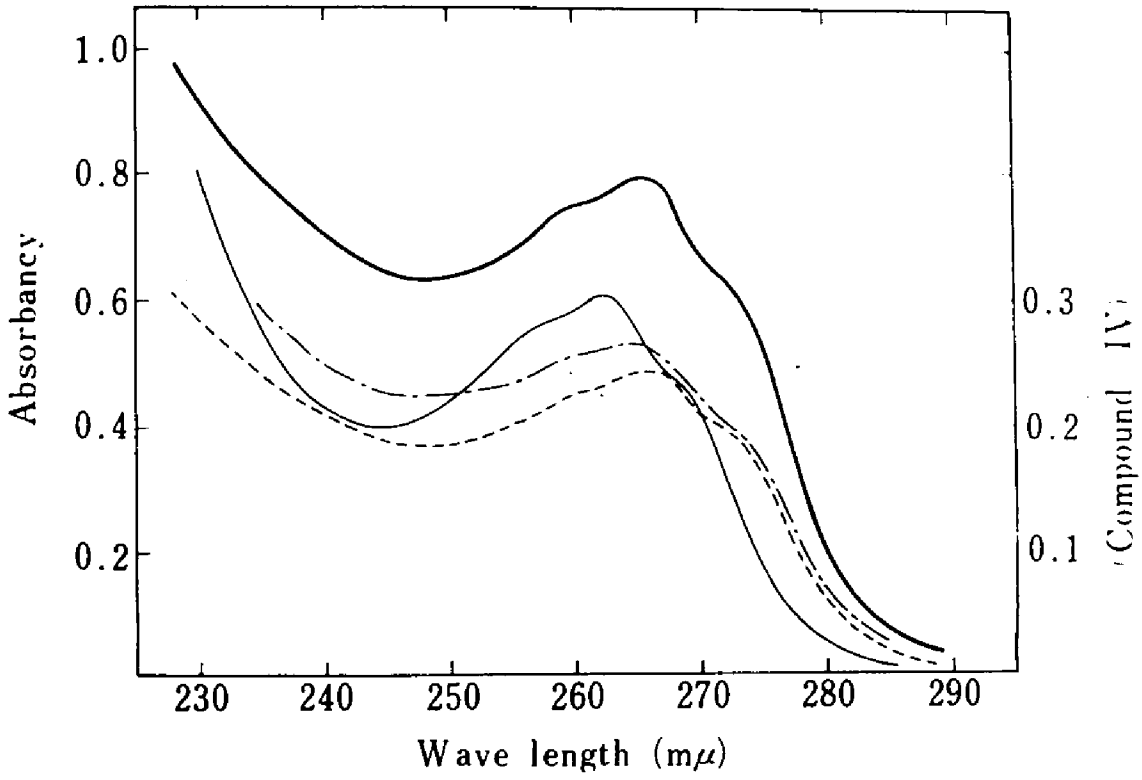

Fig. 4. Ultraviolet Absorption Spectra of Compound III, Compound IV, NMN and Nicotinamide.

——— Compound III　　—·—·— Compound IV

- - - - - NMN　　——— Nicotinamide

The concentration of each aqueous solution was 16.0 x 10^{-5} M (based on fluorometric analysis), 5.4 x 10^{-5} M (based on fluorometric analyis), 9.6 x 10^{-5} M and 9.7 x 10^{-5} M for Compound III, Compound IV, NMN and nicotinamide, respectively.

example, from 30 mg of Compound I, 4.6 mg of Compound III was obtained. This substance has an absorption maximum between 265.5 and 266 mμ and shoulders around 257 and 273 mμ (Fig. 4). The absorption spectrum did not change even when dissolved in 0.01 N hydrochloric acid instead of water. The spectrum was similar to that of NMN but different from that of nicotinamide which has an absorption maximum at 261 mμ.

IV. Analysis of chemical composition of Compounds I and III

In order to determine the chemical constituents of these compounds, quantitative analysis of each moiety and degradation by several phosphomonoesterases were carried out. The concentration of Compound I and pyridine coenzymes was calculated spectrophotometrically from molar extinction coefficients or fluorometrically, and that of Compound III was determined fluorometrically

Determination of nicotinamide. As shown in Table III, Compounds I and III contained one mole of nicotinamide per mole of each substance as expected.

Degradation by phosphomonoesterases and determination of phosphate. As shown in Table III, Compound I contained 3 moles of phosphate per mole and one of them was liberated by prostatic phosphomonoesterase; whereas Compound III contained

TABLE III. ANALYSIS OF COMPONENTS

	Nicotinamide (moles/mole of sample)	Phosphate(moles/mole of sample)		Ribose (moles/mole of sample)
		Total	Liberated by prostatic phosphatase	
NADP	1.00	3.14	0.97	
Compound I	1.00	3.03	1.09	1.95
5'-NMN	0.99	1.12	0.99	1.17(1.00)**
Compound III	1.01	2.08	1.95	0.48(1.00)**
Adenosine				1.10
5'-AMP				1.79
NAD				2.49

* The reaction mixture (1 ml) contained 1.68 μmoles of NADP, 0.91 μmole of Compound I, 1.63 μmoles of 5'-NMN or 1.60 μmoles of Compound III, 17 μmoles of $MgSO_4$, 166 μmoles of acetate buffer (pH 5.5) and prostatic phosphatase (0.65 mg protein). Incubation was carried out at 37°C for 5 hr.

** Figures in the parentheses indicate the values obtained after treatment with prostatic phosphatase.

2 moles of phosphate and all of them were liberated by the same treatment indicating that they are monoesters. These results suggest that NAD was phosphorylated to Compound I in the nicotinamide ribose moiety but not in the ribose of adenosine. The digestion products of Compounds I and III by the prostatic phosphomonoesterase were detected by paper chromatography (Table IV). NAD was formed not only from NADP, but also from Compound I.

TABLE IV. *Rf* VALUES OF DEGRADATION PRODUCTS OF COMPOUNDS I AND III TREATED WITH PROSTATIC PHOSPHATASE

	Rf values of*	
	Substrates	Products
NAD	0.22	0.22
NADP	0.11	0.22
Compound I	0.11	0.21
NMN	0.42	0.69
Compound III	0.24	0.69

Reaction system is the same as in Table III.

* Ammonium acetate-ethanol system.

TABLE V. FORMATION OF NAD FROM COMPOUND I BY PROSTATIC PHOSPHATASE

Substrate	Substrate added (μmoles)	NAD formed (μmoles)	
		determined by alcohol dehydrogenase*	determined by paper chromatography and fluorometry
NADP	1.82	1.37	1.32**
Compound I	1.70	1.44	1.40

The reaction mixture (1 ml) contained substrate, 17 μmoles of $MgSO_4$, 166 μmoles of acetate buffer (pH 5.5) and prostatic phosphatase (0.65 mg protein). Incubation was carried out at 37°C for 4 hr.

* NAD was determined using the following reaction system: pyrophosphate-semicarbazide-glycine buffer (pH 8.8), 2.0 ml; 1.74 M ethanol, 0.2 ml; reaction mixture stated above, 0.2 ml; alcohol dehydrogenase, 30 μg; total volume 3.0 ml. NAD was calculated from absorbancy at 340 mμ of reduced NAD.

** Remainder of NADP, 0.46 μmole.

TABLE VI. DEGRADATION OF COMPOUNDS I AND III BY NUCLEOTIDASES

(A) Degradation by seminal 5'-nucleotidase

Substrate	Substrate added (μmoles)	Substrate remaining (μmoles)	NAm-R formed (μmoles)
5'-NMN	1.68	0	1.46
Compound III	1.18	1.17	0

The reaction mixture (1.4 ml) contained substrate, 5 μmoles of $MgSO_4$, 50 μmoles of Tris-HCl buffer (pH 8.6), and 5'-nucleotidase (0.94 mg protein). Incubation was carried out at 37°C for 1 hr.

(B) Degradation by 3'-nucleotidase

Substrate	Substrate added (μmoles)	Phosphate liberated(μmole) by the enzyme from	
		Wheat	*B. subtilis*
5'-NMN	1.22	0	0.06
Compound I	1.23	0	0
Compound III	1.20	0.06	0

The reaction mixture (1.4 ml) contained substrate, 75 μmoles of Tris-HCl buffer (pH 8.0) and wheat enzyme (0.27 mg protein) or *B. subtilis* enzyme (0.15 mg protein). Incubation was carried out at 37°C for 1 hr.

Determination of substrate and nicotinamide riboside was carried out by paper chromatography in an ammonium acetate-ethanol system and fluorometry.

This was also demonstrated with alcohol dehydrogenase as shown in Table V. The incubation of Compound III with the prostatic phosphatase gave one mole of nicotinamide riboside and two moles of phosphate. In order to decide the position of phosphomonoester bond of Compounds I and III, the action of 3'- and 5'-nucleotidases on the compounds was investigated. Results are shown in Table VI. By seminal 5'-nucleotidase, NMN was degraded to nicotinamide riboside, whereas Compound III was not. Since seminal 5'-nucleotidase is known to have little activity on 3', 5'-diphosphate or 2',5'-diphosphate,[84] there is a possibility that a phosphoryl group exists at 2' or 3' position besides the 5' position of hydroxyl groups of the nicotinamide riboside. Therefore, the degradation by 3'-nucleotidases was examined. By the action of nucleotidase preparations of wheat and *B.subtilis*, neither Compound I nor III was degraded.

Determination of ribose. The amounts of ribose in Compounds I and III were tentatively determined by the orcinol method. One mole of Compound I gave rise to 1.95 moles of ribose; on the other hand, in case of Compound III, only 0.48 mole of ribose was detected. It is known that the intensity of color development in the orcinol method is greatly influenced by the binding of phosphates or the position of phosphate linkage in ribose moiety to be tested.[69,85,86] Therefore, ribose

moiety of NMN and Compound III was assayed after the digestion by prostatic phosphatase. As presented in Table III, the same amounts of ribose was detected both in NMN and Compound III.

V. Phosphorylation of 5'-NMN

Isolation of the reaction product. As mentioned above, phosphorylation of NAD by the bacterial transferring system might occure at 2'- or 3'-position of the nicotinamide ribose moiety in NAD. Therefore, phosphorylation of 5'-NMN was investigated in the following way. Incubation was carried out with a reaction mixture containing 10 μmoles of 5'-NMN, 40 μmoles of *p*-NPP, 20 μmoles of nicotinamide, 1 μmole of zinc sulfate and ammonium sulfate fraction of *P. mirabilis* (4.5 mg as protein) in a total volume of 1 ml. As a control experiment, 5'-NMN was incubated without *p*-NPP. After 2 hr incubation at 37°C, the reaction was stopped by heating in boiling water for 3 min and the precipitated protein was removed by centrifugation. An aliquot of 0.025 ml was spotted on a filter paper and developed in isobutyric acid-ammonia system. In the reaction system containing *p*-NPP, a new spot (Compound IV) which showed fluorescence by the treatment with a mixed vapor of methyl ethyl ketone and ammonia was developed below NMN on the paper. The amount of the Compound IV (4.1 μmoles per ml of the reaction mixture) was determined

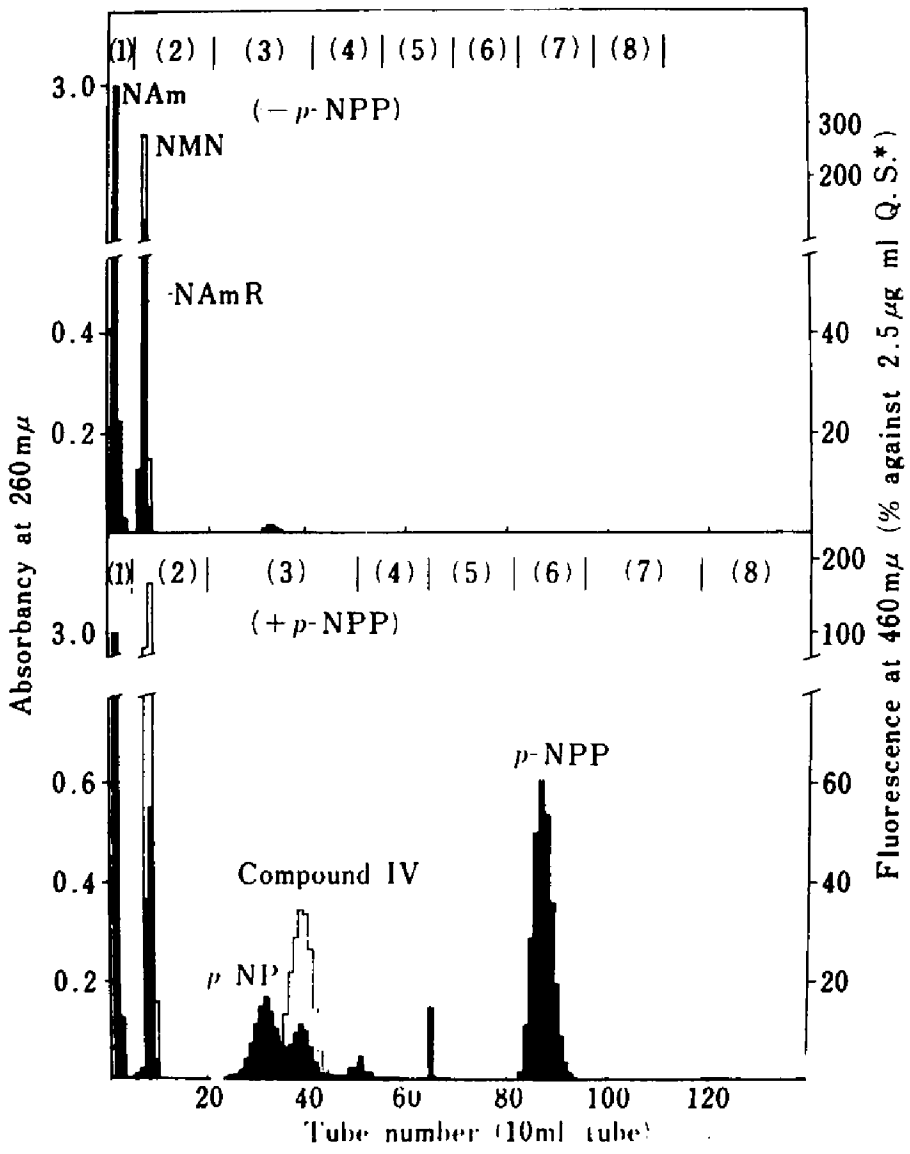

Fig. 5. Separation of Compound IV by Column Chromatography.

Absorbancy Fluorescence

Column: Dowex 1 x 2, formate form, 0.79 cm^2 x 14.0 cm.

(1) Water
(2) 0.01 M Formic acid
(3) 0.05 M Formate buffer
(4) 0.2 M Formate buffer
(5) 0.5 M Formate buffer
(6) 1.0 M Formate buffer
(7) 2.0 M Formate buffer
(8) 4.0 M Formate buffer

*0.1 N sulfuric acid solution of quinine sulfate.

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fluorometrically. Then, 0.5 ml of the reaction mixture was applied on a Dowex 1 x 2 (formate form) column and eluted with formic acid and formate buffer. The elution patterns are presented in Fig. 5, which shows that Compound IV can be eluted with 0.05 M buffer. This fraction was collected, adsorbed to 100 mg active charcoal and then extracted with 50 ml of pyridine-ethanol-water mixture. The extract was dried up with a rotary evaporator and the residue was dissolved in 1 ml of water. To remove a small amount of contaminating *p*-NP, the solution was washed 4 times with 4 ml of ethyl acetate and twice with 2 ml of ethyl ether. The aqueous layer was diluted and subjected to spectral analysis. Figure 4 shows that the absorption spectrum of Compound IV is very similar to that of Compound III and 5'-NMN and has a maximum at 265 mμ.

The behaviors of Compound IV were investigated using paper chromatography and high voltage paper electrophoresis. The results are shown in Table II. Compounds III and IV have the same *Rf* values in three different solvent systems and the same

mobilities in paper electrophoresis. Higher mobility of these compounds to anode comparing with that of NMN suggested that these compounds had the higher negative charge which might be caused by the second phosphoryl group introduced to NMN.

Degradation of Compound IV by nucleotidases. In order to determine the numbers and the position of phosphoric ester linkage, degradation of Compound IV was carried out with several phosphomonoesterases. Compound IV used in this experiment was prepared from 5'-NMN using the reaction system mentioned above, except that nicotinamide was omitted. The results are shown in Table VII. Since 2 moles of phosphate were detected per mole of Compound IV with the nonspecific phosphatase (a little or no liberation of phosphate took place with either 5'- or 3'-nucleotidase), all of the phosphoryl groupings were of monoester linkage. These results agree well with the facts obtained from the similar experiments with Compound III.

DISCUSSION

This section demonstrated that the phosphorylation of NAD by the enzyme preparation from *P. mirabilis* gave rise to at least two compounds when *p*-NPP was used as a phosphoryl donor. There are two possible positions in the ribose of NAD to which the phosphate of *p*-NPP or nucleoside monophosphate may be introduced.

TABLE VII. DEGRADATION OF COMPOUND IV BY PHOSPHOMONOESTERASES

Substrate*1	Total phosphate (moles/mole of substrate)	Phosphate liberated (moles/mole of substrate) by		
		Prostatic phosphatase*2	5'-Nucleotidase*3	3'-Nucleotidase*4
Compound IV	1.85	1.75	0	0.05
5'-NMN	0.96	1.00	0.77	0.04

* 1 Concentration of substrate was determined fluorometrically.

* 2 The reaction mixture (1 ml) contained 0.48 μmole of Compound IV or 0.96 μmole of NMN, 10 μmoles of $MgSO_4$, 200 μmoles of acetate buffer (pH 5.5) and prostatic phosphatase (0.2 mg protein). Incubation was carried out at 37°C for 2.5 hr.

* 3 The reaction mixture (1 ml) contained 0.96 μmole of Compound IV or 0.96 μmole of NMN, 10 μmoles of $MgSO_4$, 100 μmoles of Tris-HCl buffer (pH 8.5) and 5'-nucleotidase (0.9 mg protein). Incubation was carried out at 37°C for 2 hr.

* 4 The reaction mixture (1 ml) contained 0.96 μmole of Compound IV or 0.96 μmole of NMN, 100 μmoles of Tris-HCl buffer (pH 7.2) and 3'-nucleotidase from *B. subtilis* (0.8 mg protein). Incubation was carried out at 37°C for 2 hr.

NADP is formed by phosphotransferring reaction to 2' position of the adenosine of NAD. Shuster[87] reported that 2'-phosphate of NADP migrated to 3' position in acidic conditions. On the other hand, Mitsugi *et al.*[51, 52] reported that the phosphate of *p*-NPP was transferred to 2' or 3' position of adenosine and 5'-AMP by the nucleoside phosphotransferase of bacteria including *P. mirabilis*. However, in the present experiments any conclusive data

on 3'-NADP formation was obtained. There is another possibility that the phosphate of *p*-NPP or nucleoside monophosphate may be transferred to 2' or 3' position of nicotinamide ribose of NAD. Although Compound I showed the same behaviors as NADP in electrophoresis and chemical analysis of the constituents, its degradation products by snake venom was apparently different from those of NADP. With the preparation from Mamushi venom, NADP was degraded to adenosine 2',5'-diphosphate and NMN, and subsequently the latter was dephosphorylated to nicotinamide riboside. Nicotinamide might be formed by the successive action of nucleotidase and nucleosidase after the degradation of pyrophosphate bond of NAD, because of the absence of NADase[88] in the venom preparation. On the other hand, after the degradation of Compound I with lyophylized snake venom or the purified nucleotide pyrophosphatase, Compound III, which was not found in the degradation products of NADP, was formed besides adenosine or AMP and a small amount of nicotinamide.

It is obvious that, of the two phosphates of Compound III, the one is located at 5' position, since this substance was formed by the action of nucleotide pyrophosphatase. However, this phosphate was not liberated by seminal 5'-nucleotidase. Presence of another phosphate is also suggested by the fact that 5'-nucleotidases of animal origin have no activity on nucleoside 2',5'-

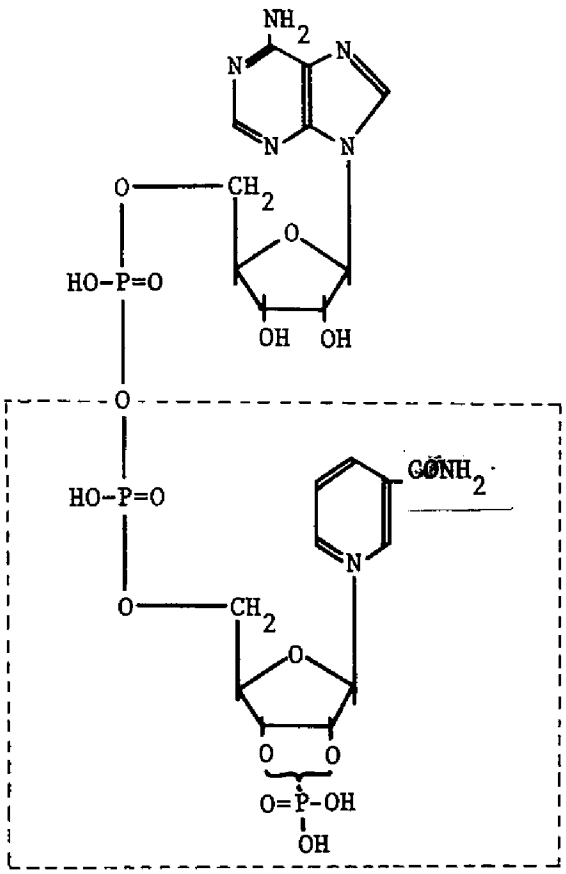

Fig. 6. Structure of Compound I (NADP Analog).

The part surrounded by a broken line indicates the structure of Compound III or IV (nicotinamide riboside diphosphate).

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or 3',5'-diphosphate.[81,84] The result that the liberation of phosphate did not occure by 3'-nucleotidase obtained from wheat or *B. subtilis* suggested the occurrence of the phosphate bond at 2' position of nicotinamide ribose.

On the other hand, 5'-NMN was phosphorylated by the same enzyme preparation of *P. mirabilis* to form Compound IV. This substance showed similar properties to Compound III in column chromatography, paper chromatography and high voltage paper electrophoresis. One mole of Compound IV liberated 2 moles of phosphate by nonspecific phosphomonoesterase but no phosphate by 5'- and 3'-nucleotidases. These results suggested that Compound III and Compound IV were the same substance, namely nicotonamide ribose diphosphate.

The problem whether the phosphate linkage other than that in 5' position is really located in 2' position, and therefore not in 3' position should be determined by the other chemical procedures. Based on the results obtained in the present experiments, structures of Compounds I and III (or IV) may be shown as in Fig. 6.

SUMMARY

A new derivative of NAD (Compound I) synthesized from NAD and *p*-NPP by the enzyme preparation of *Proteus mirabilis* (IFO 3849), was isolated from the reaction mixture.

After degradation of this compound with snake venom nucleotide pyrophosphatase, Compound III was obtained. 5'-NMN was phosphorylated to Compound IV by the same enzyme preparation

of *P. mirabilis*. By the determination of chemical constituents and the degradation with phosphomonoesterases, Compounds III and IV were identified as nicotinamide riboside 2' (3'), 5'-diphosphate, and Compound I was identified as NADP analog which was formed by phosphorylation at the 2' or 3' position of the nicotinamide ribose moiety, not at the 2' position of adenosine moiety of NAD.

Section 3. Phosphorylation of NADP and NADP Analog

INTRODUCTION

It was reported in the previous chapters that when NAD and *p*-NPP were incubated with the enzyme preparation of *Proteus mirabilis* (IFO 3849) under acidic conditions, NADP and NADP analog, a new NAD derivative, were synthesized. Biochemical analysis demonstrated that in the structure of NADP analog the phosphoryl group introduced was located at 2' (or 3') position in the nicotinamide ribose moiety. Nicotinamide riboside 2' (or 3'),5'-diphosphate, which was given after the degradation of NADP analog with nucleotide pyrophosphatase, was formed also by the phosphorylation of 5'-NMN.

This section deals with the phosphorylation of NADP or NADP analog and identification of the products.

MATERIALS AND METHODS

Culture of Proteus mirabilis and preparation of the enzyme solution. Culture of *P. mirabilis* (IFO 3849) and the preparation of ammonium sulfate fraction of enzyme solution were carried out according to the methods described in Section 1.

Standard reaction system. The phosphotransferring reaction was investigated under the standard reaction system con-

taining 7 - 10 μmoles of phosphoryl acceptor, 40 μmoles of *p*-NPP, 20 μmoles of nicotinamide, 1 μmole of zinc sulfate, 300 μmoles of acetate buffer (pH 4.0) and 5 mg of enzyme protein per ml of the mixture. After incubation at 37°C, the reaction was stopped by heating the mixture in a boiling water bath for 3 min and the precipitate formed was centrifuged off.

Separation of pyridine coenzymes. An aliquot of the supernatant solution of the reaction mixture was placed on Toyo filter paper No 53 and paper chromatography by the descending technique was performed with several solvent systems: (a) isobutyric acid : 0.5 N ammonium hydroxide = 10 : 6 [55] (b) 95% ethanol : 1 M ammonium acetate = 7 : 3 (pH 5.0)[64] (c) saturated ammonium sulfate solution : 0.1 M sodium acetate : isopropanol = 79 : 19 : 2.[65] Isobutyric acid system was rather satisfactory for the separation of NAD and its derivatives, and employed for the determination of these compounds. High voltage paper electrophoresis was performed with both buffer systems of 0.2 M acetate (pH 3.5) and 0.1 M borate (pH 9.4). Pyridine nucleotides were distinguished from other base derivatives by development of fluorescent spot on methyl ethyl ketone treatment.[59] Column chromatography using Dowex 1 x 2 (formate form) was carried out to isolate the reaction products.

Analysis. Pyridine nucleotides in the reaction mixture

was separated by paper chromatography with the isobutyric acid system. Substances on the paper were detected by Manasuru UV lamp. Each spot corresponding to pyridine nucleotides were cut off and extracted with 0.01 N hydrochloric acid and determined fluorometrically according to the methyl ethyl ketone method using quinine sulfate as the standard of fluorescence. Nicotinamide riboside linkage in the chromatographic fractions was also determined fluorometrically. Concentration of NAD and NMN derivatives in the solution was also determined fluorometrically using NADP as the standard specimen. These fluorometric techniques are described in the previous sections. Conveniently the molar extinction coefficients for NADP (18.0×10^6 cm^2 at 260 mμ) and NMN (4.6×10^6 cm^2 at 266 mμ) were used tentatively for the calculation of the concentration of NAD and NMN derivatives respectively, because the coefficients for these derivatives obtained in this experiments were not decided. Total phosphate was assayed by determing the inorganic phosphate after digestion of the sample with sulfuric acid according to the method of Fiske-SubbaRow.[66] Phosphate liberated by phosphomonoesterases was also determined by this method. Protein was determined by the method of Lowry.[70]

Spectral analysis of NADP derivatives was performed by Shimazu Multipurpose Spectrophotometer Model 50L.

Nucleotides. NADP analog and nicotinamide riboside 2'(3'),5'-diphosphate were obtained by the enzymic phosphorylation of NAD and 5'-NMN respectively as described in the previous section. Adenosine 2'(3'),5'-diphosphate was prepared by the phosphorylation of 5'-AMP according to the method of Mitsugi *et al.*[52] using cell-free extract of *Proteus mirabilis* as the enzyme preparation.

Enzymes. Lyophilyzed powder of Mamushi (*Agkistrodon halys blomhoffii*) venom was employed as the preparation of nucleotide pyrophosphatase.[76] Alkaline phosphatase of calf intestine (degree of purity I) was obtained from C. F. Boehringer and Soehen GmbH. 3'-Nucleotidase from culture broth of *Bacillus subtilis* was kindly presented by Dr. Sugino of Institute for Virus Research, Kyoto University.

RESULTS AND DISCUSSION

Enzymic synthesis of new NAD derivatives. When NADP was incubated with the enzyme preparation of *P. mirabilis* under the standard reaction system, a product (Compound V) was synthesized which migrated slower than NADP by the paper chromatography with the isobutyric acid system and gave fluorescence on treatment with methyl ethyl ketone-ammonia vapor (Fig. 1). When NADP analog was incubated with the enzyme, a product (Compound VI) was formed

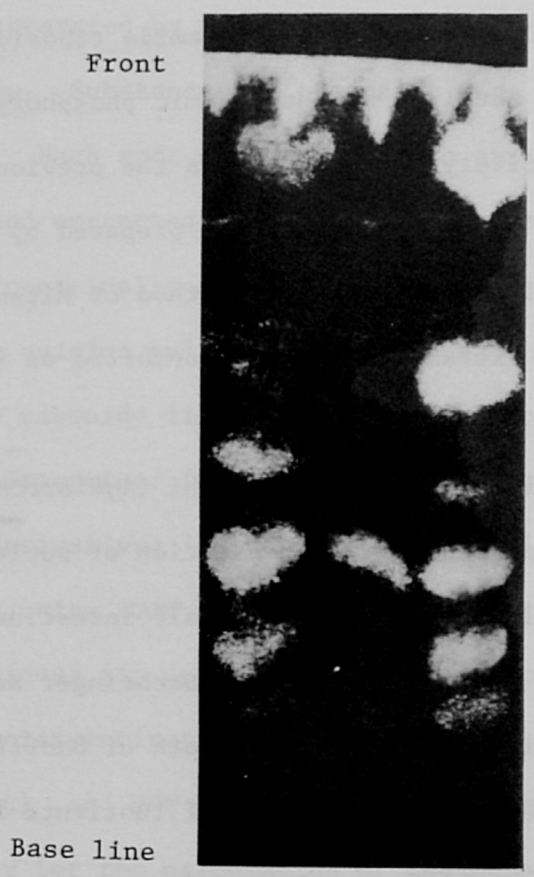

Fig. 1. Separation of Reaction Products by Paper Chromatography with Isobutyric Acid-Ammonia System.

Parentheses indicate the spot which showed fluorescence after methyl ethyl ketone treatment.

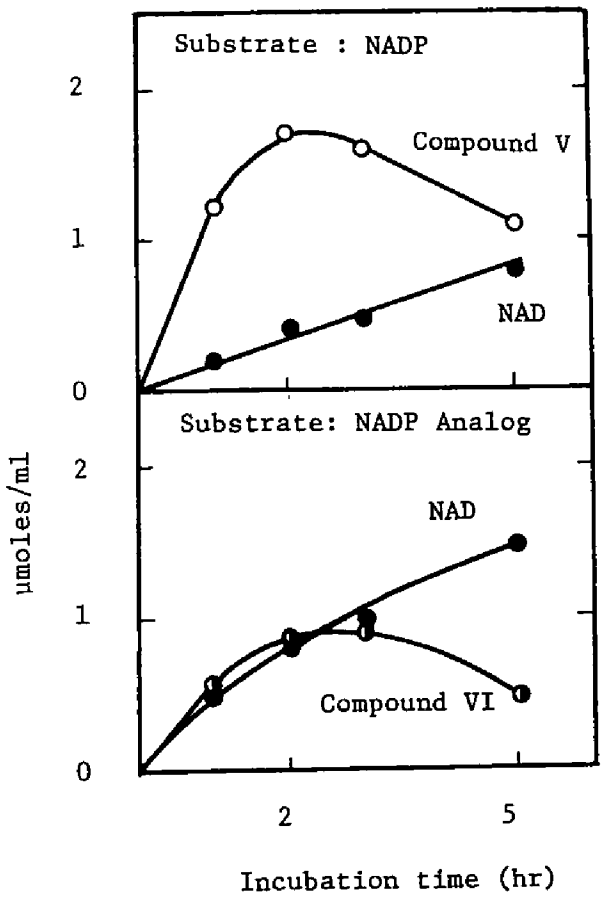

Fig. 2. Time Course of Compounds V and VI Formation.

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which showed the similar behavior to Compound V. Aliquots of the reaction mixture were taken out hourly and subjected to quantitative analysis of the products. Amount of these compounds was determined fluorometrically as NADP-derivatives. Time course of the formation of these compounds is shown in Fig. 2. After

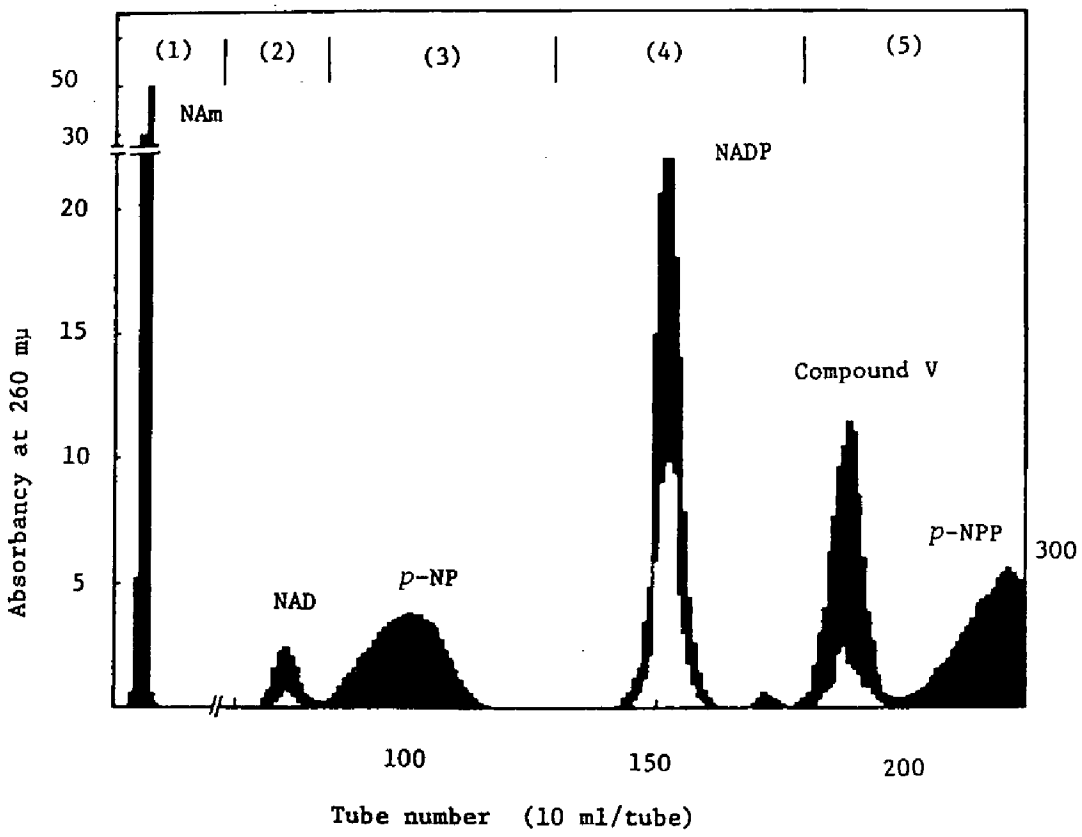

Fig. 3. Separation of Compound V by Column Chromatography on Dowex 1.

■ Absorbancy □ Fluorescence

The supernatant solution of the reaction mixture was applied to a column of Dowex 1 x 2 (formate form, 2.5 cm^2 x 18 cm).

(1) Water
(2) 0.05 M Formate buffer
(3) 0.1 M Formate buffer
(4) 0.5 M Formate buffer
(5) 1.0 M Formate buffer

* 0.1 N sulfuric acid solution of quinine sulfate

2 hr incubation, about 28% of NADP was converted to Compound V, whereas only 16% of NADP analog was to Compound VI. In order to obtain sufficient quantities of Compound V for the investigation on chemical properties, relatively large scale reactive mixture was used containing 200 μmoles of NADP, 800 μmoles of *p*-NPP, 400 μmoles of nicotinamide, 20 μmoles of zinc sulfate, 6 mmoles of acetate buffer (pH 4.0) and 96 mg of enzyme protein of ammonium sulfate fraction in a total volume of 20 ml. The incubation was carried out for 4 hr at 37°C Four hundreds μmoles *p*-NPP were added after 2 hr incubation in order to prevent the degradation of the product. The reaction was stopped by boiling the reaction mixture for 5 min and the precipitate formed was centrifuged off. The supernatant solution was adjusted to pH 6.8 with ammonium hydroxide and introduced on a Dowex 1 x 2 (formate form) column. Stepwise elution with increasing concentrations of formate buffer (pH 3.4) was carried out. The elution pattern is presented in Fig. 3. Compound V was eluted with 1.0 M buffer in a single peak and detected fluorometrically. This material in the fraction was adsorbed on 400 mg of active charcoal, and was extracted from the charcoal with 200 ml of pyridine-ethanol-water (10 : 45 : 45) mixture. The extracts were concentrated by a rotary evaporator under reduced pressure. By repeating of precipitation from cold acetone, 21.7 mg of white powder of

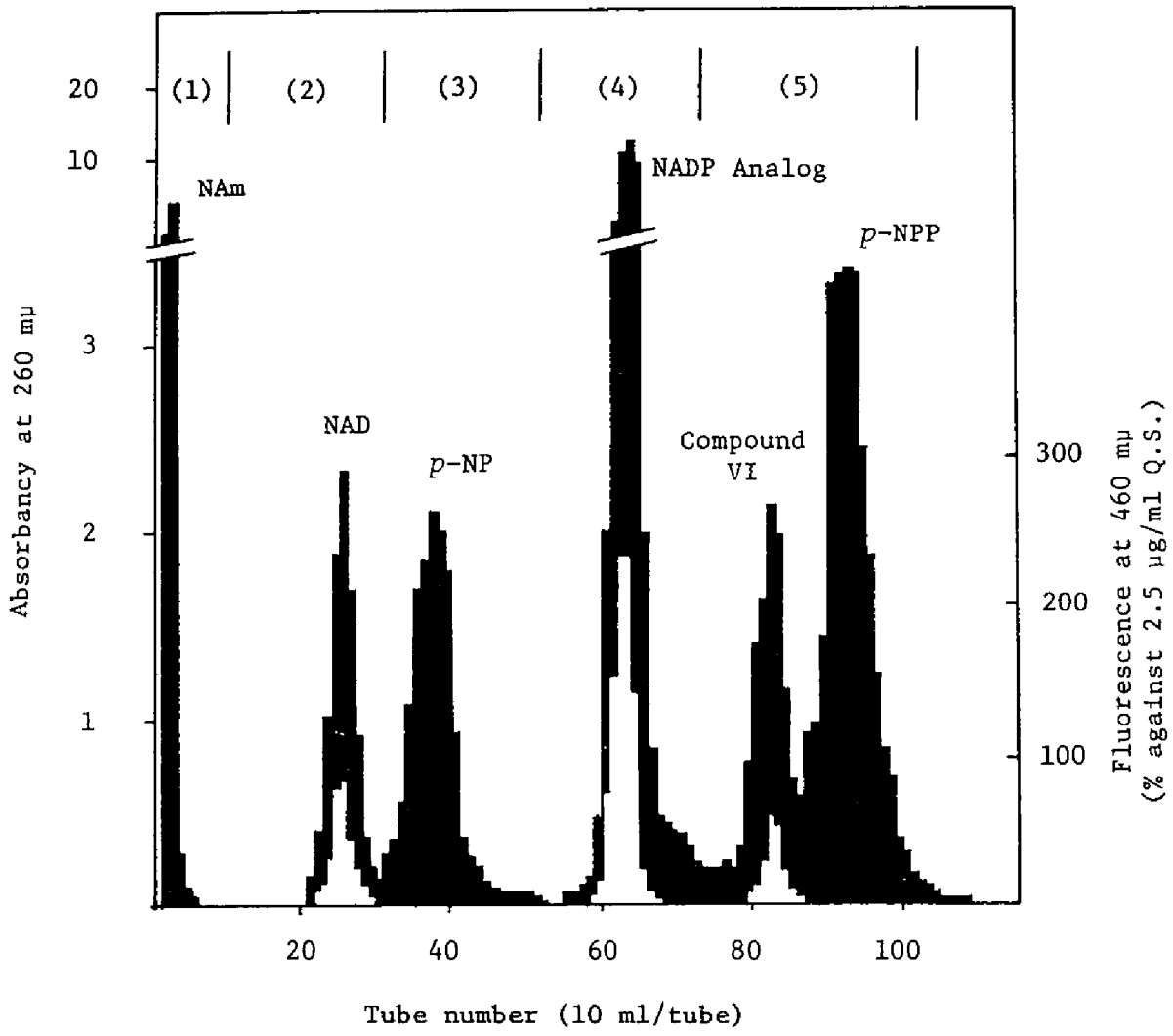

Fig. 4. Separation of Compound VI by Column Chromatography on Dowex 1.

Absorbancy Fluorescence

The reaction mixture (4 ml) contained 40 μmoles of NADP analog, 160 μmoles of *p*-NPP, 80 μmoles of nicotinamide, 4 μmoles of zinc sulfate, 1.2 mmoles of acetate buffer (pH 4.0), and ammonium sulfate fraction (19.2 mg protein). The incubation was carried out

at 37°C for 4 hr. Eighty μmoles of *p*-NPP was added after 2 hr. incubation. The supernatant solution was applied to a column of Dowex 1 x 2 (formate form, 0.87 cm^2 x 18.5 cm).

(1) Water
(2) 0.05 M Formate buffer
(3) 0.1 M Formate buffer
(4) 0.5 M Formate buffer
(5) 1.0 M Formate buffer

Compound V was obtained. From the reaction mixture containing NADP analog as the substrate, 1.8 μmoles of Compound VI was isolated by the same prodedure as in Compound V (Fig. 4) but this compound was obtained as an aqueous solution.

Determination of total phosphate and degradation by phosphomonoesterases. As shown in Table I (Experiment 1), 4 moles of phosphate were detected per mole of Compound V and VI. The incubation of these compounds with phosphomoesterase (alkaline phosphatase) gave two moles of phosphates. This result suggests that NADP and NADP analog were phosphorylated to NAD diphosphate (Compound V) and NADP analog phosphate (Compound VI) respectively, and phosphoryl group introduced was of monoester linkage. The fact that a portion of the phosphates was liberated by the incubation of Compound VI with 3'-nucleotidase indicates that this compound contains 3'-phosphoryl group in the molecular structure.

Spectral analysis. Spectral properties of Compounds V

TABLE I. DEGRADATION OF COMPOUNDS V, VI, A, B, C AND D BY PHOSPHOMONOESTERASES

Experiment No.	Substrate*1	Total phosphate (moles/mole of substrate)	Phosphate liberated (moles/mole of substrate) by	
			alkaline phosphatase*2	3'-Nucleotidase*3
1	Compound V	3.85	1.82	0.09
	Compound VI	3.89	1.85	0.24
	NADP	3.02	0.97	-
2	Compound A	1.97	1.82	0.01
	Compound B	1.99	1.80	0.03
	Compound C	1.87	1.85	0.03
	Compound D	2.07	1.91	0.28
	3'-AMP	-	0.96	0.95

*1) Concentrations of Compounds V, VI, A and B were determined fluorometrically using NADP as standard specimen. Concentrations of Compounds C and D was calculated using molar extinction coefficient for ADP, 15 x $10^6 cm^2$ at 260 mμ.

*2) Experiment 1: The reaction mixture (0.5 ml) contained 0.28 μmole of Compound V, 0.27 μmole of Compound VI or 0.53 μmole of NADP, 50 μmoles of glycine-NaOH buffer (pH 10.5), 0.5 μmole of $MgCl_2$, 0.05 μmole of $ZnSO_4$ and enzyme (0.8 μg protein). Incubation was carried out at 37°C for 30 min.

Experiment 2: The reaction mixture (1.55 ml) contained 0.85 μmole of Compound A, 0.74 μmole of Compound B, 0.82 μmole of Compound C, 0.61 μmole of Compound D or 1.43 μmoles of 3'-AMP, 150 μmoles of glycine-NaOH buffer (pH 10.5), 1.5 μmoles of $MgCl_2$, 0.15 μmole of $ZnSO_4$ and enzyme (50 μg protein).

Incubation was carried out at 37°C for 1 hr.

*3) Experiment 1: The reaction mixture (1 ml) contained 0.83 μmole of Compound V or 0.81 μmole of Compound VI, 150 μmoles of Tris-HCl buffer (pH 7.2) and enzyme (0.7 mg protein). Incubation was carried out at 37°C for 4 hr.
Experiment 2: The reaction mixture (1.5 ml) contained 1.70 μmoles of Compound A, 1.47 μmoles of Compound B, 0.82 μmole of Compound C, 0.88 μmole of Compound D or 1.43 μmoles of 3'-AMP, 150 μmoles of Tris-HCl buffer (pH 7.2) and enzyme (0.14 mg protein). Incubation was carried out at 37°C for 3 hr.

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and VI in aqueous solution were examined. As shown in Fig. 5, absorption spectra of these compounds were identical with that of pyridine coenzymes such as NADP and showed maximum at 259 mμ.

Behaviors in paper chromatography and high voltage paper electrophoresis. As presented in Table II, the compounds isolated here showed similar behaviors with three solvent systems. In electrophoresis with two buffer systems, Compounds V and VI migrated much more rapidly to the anode than NADP or NADP analog owing to the higher electro-negativity brought about by the phosphoryl group introduced.

Degradation of Compounds V and VI. In order to investigate chemical structures of Compound V and VI, degradation by snake venom nucleotide pyrophosphatase was examined (see Fig. 6). After the incubation, the supernatant solution of each

TABLE II. BEHAVIORS OF COMPOUNDS V, VI, A, B, C AND D, IN

Compound	*Rf* values: Isobutyric acid-ammonia	*Rf* values: Am-acetate ethanol
NADP	0.38	0.02
NADP analog	0.40	0.02
Compound V	0.24	0.01
Compound VI	0.26	0.01
5'-NMN	0.51	0.28
Nicotinamide riboside diphosphate	0.31	0.09
Adenosine 2'(3'),5'-diphosphate	0.41	0.09
Degradation products from Compound V — Compound A	0.32	0.09
Degradation products from Compound V — Compound C	0.38	0.10
Degradation products from Compound VI — Compound B	0.31	0.09
Degradation products from Compound VI — Compound D	0.41	0.09

* indicate migration distance in centimeter to the anode

reaction mixture was adjusted to pH 6.8 with ammonium hydroxide and applied to the column of Dowex 1 x 2 (formate form). Stepwise elution with formate buffer (pH 3.5) was performed as presented in Fig. 6. With 0.05 M formate buffer, the degradation product which revealed fluorescence by the methyl ethyl ketone

PAPER CHROMATOGRAPHY AND HIGH VOLTAGE PAPER ELECTROPHORESIS

Am-sulfate-Na-acetate isopropanol	Mobilities*	
	0.2 M acetate buffer, pH 3.5 3KV, 15mA, 50min	0.1 M borate buffer, pH 9.4 3KV, 25mA, 30min
0.32	14.5	16.0
0.40	14.5	15.5
0.54	25.5	18.5
0.52	26.0	18.5
0.78	0.5	14.5
0.82	16.5	20.5
0.40	20.0	18.5
0.81	16.5	20.5
0.44	20.0	18.5
0.82	16.5	20.5
0.39	20.0	18.0

method was eluted (Compound A or B). This compound was adsorbed to active charcoal and extracted with pyridine-ethanol-water mixture. The extract was concentrated under reduced pressure and the syrup was dissolved in a small amount of water. With 0.5 M buffer, the substance which has absorption maximum at

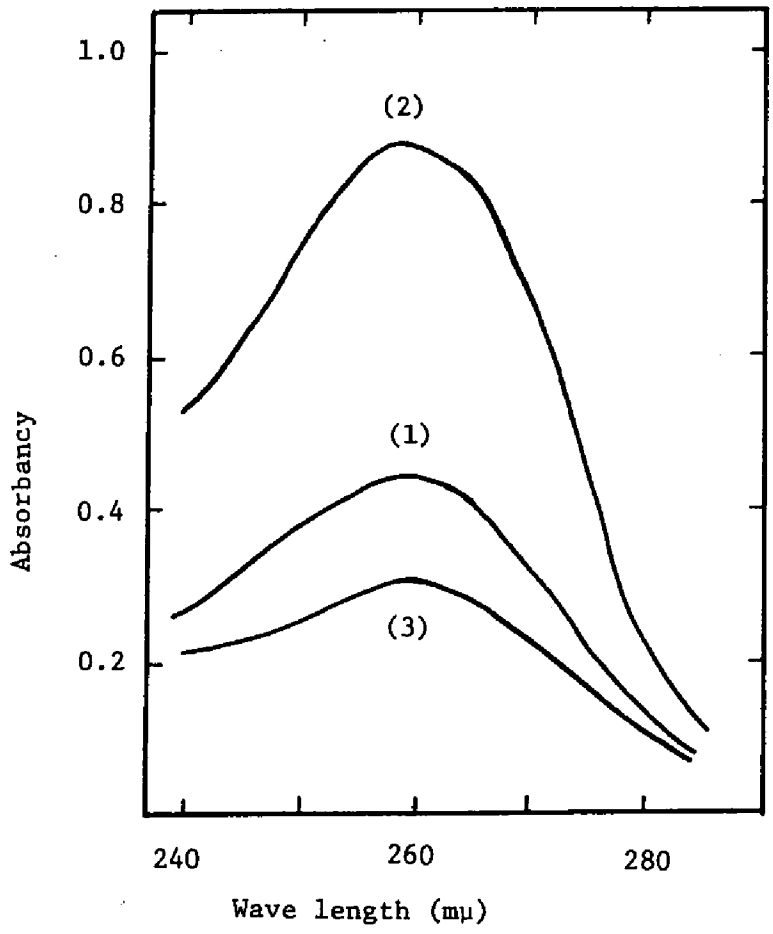

Fig. 5. Ultraviolet Absorption Spectra of Compounds V and VI.

(1) NADP (2.5×10^{-5} M)

(2) NAD diphosphate (Compound V) (4.9×10^{-5} M)

(3) NADP analog phosphate (Compound VI) (1.7×10^{-5} M)

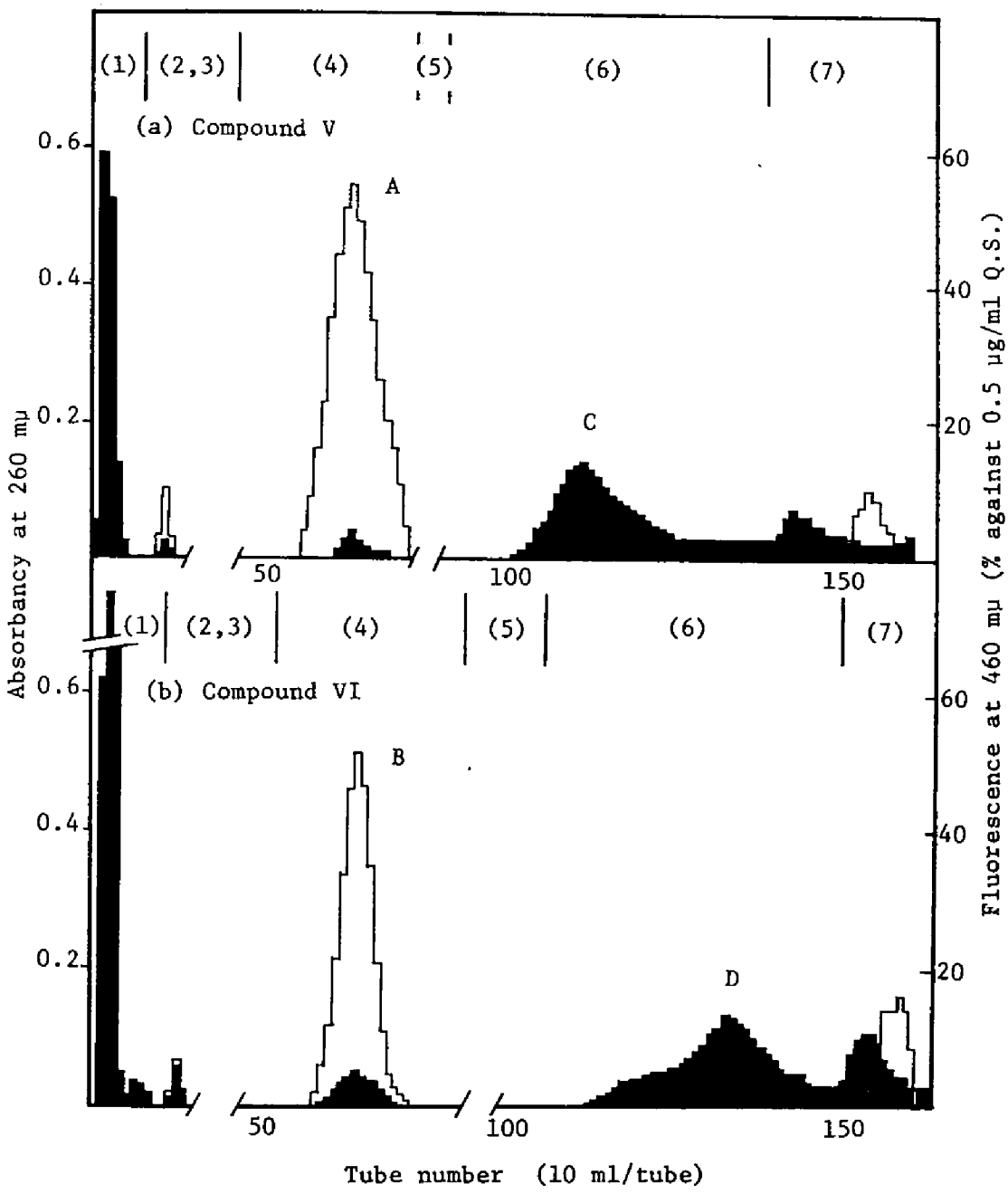

Fig. 6. Degradation of Compounds V and VI by Snake Venom.

Absorbancy Fluorescence

(a) The reaction mixture (2 ml) contained 2.5 μmoles of Compound V, 250 μmoles of Tris-HCl buffer (pH 7.2), and 2.5 mg of lyophylized powder of snake venom. Incubation was carried out at 37°C for 16 hr. The supernatant solution was applied to a column of Dowex 1 x 2 (formate form, 0.78 cm^2 x 12.5 cm).

(b) The reaction mixture (3 ml) contained 2.2 μmoles of Compound VI, 385 μmoles of Tris-HCl buffer (pH 7.2), and 3.75 mg of lyophylized powder of snake venom. Incubation was carried out at 37°C for 16 hr. The supernatant solution was applied to a column of Dowex 1 x 2 (formate form, 0.78 cm^2 x 17 cm).

(1) Water
(2) 0.01 M Formic acid
(3) 0.02 M Formate buffer
(4) 0.05 M Formate buffer
(5) 0.1 M Formate buffer
(6) 0.5 M Formate buffer
(7) 1.0 M Formate buffer

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259 mμ was eluted (Compound C or D). This substance was also obtained by treatment with active charcoal, elution with pyridine-ethanol-water mixture and condensation under reduced pressure.

Each aliquot of the solutions obtained above was subjected to paper chromatography and high voltage paper electrophoresis. The degradation products which were eluted with 0.05 M (Compounds A and B) and 0.5 M (Compounds C and D) buffer have the same mobility as those of nicotinamide riboside diphosphate and adenosine 2'(3'),5'-diphosphate respectively (Table II). UV spectra of the degradation products in 0.01 N hydrochloric acid are presented in Fig. 7. Compounds A and B have absorption maximum

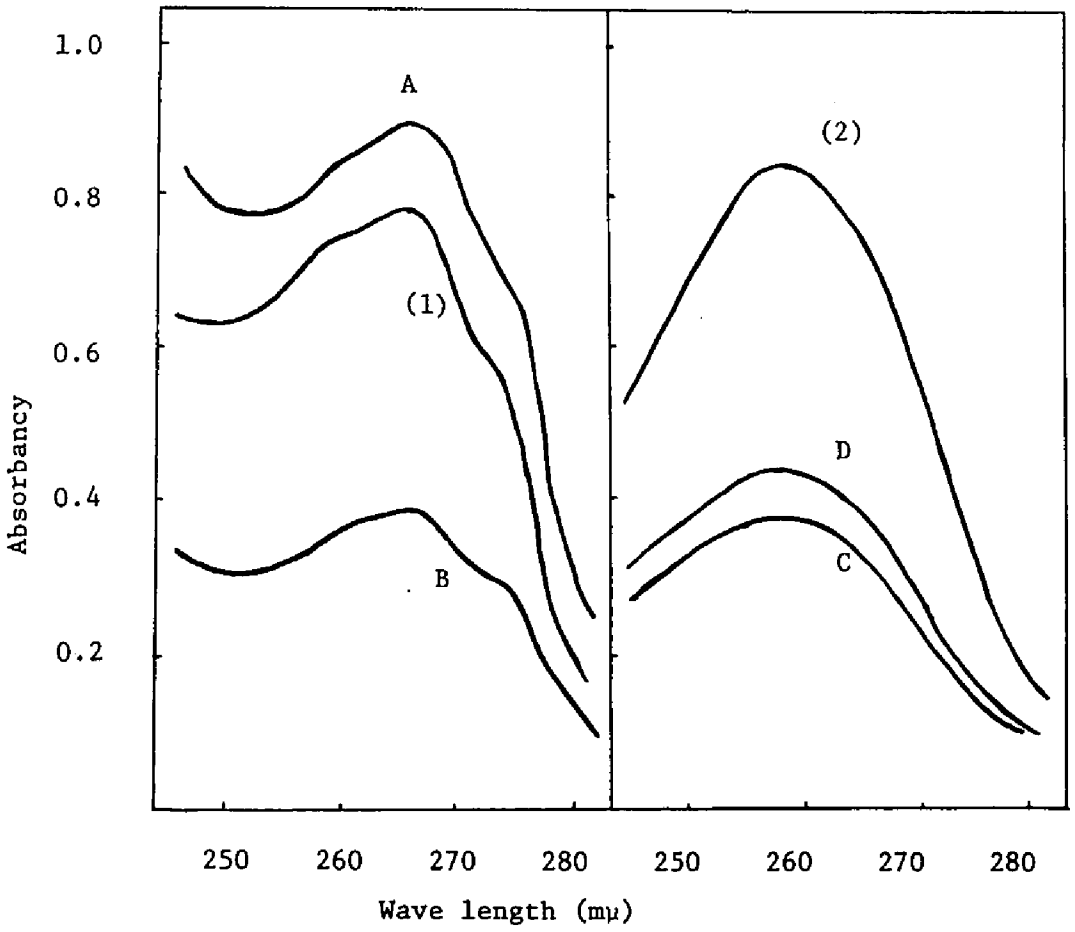

Fig. 7. Ultraviolet Absorption Spectra of Degradation Products.

A, 2.0×10^{-4} M

B, 0.97×10^{-4} M

(1), nicotinamide riboside diphosphate, 1.7×10^{-4} M

C, 2.7×10^{-5} M

D, 3.1×10^{-5} M

(2), adenosine 2'(3'),5'-diphosphate, 5.8×10^{-5} M

around 266 mμ, similar with that of nicotinamide riboside diphosphate. On the other hand, Compounds C and D have the same spectral property as that of adenosine 2'(3'),5'-diphosphate.

Other baches of Compounds A, B, C and D were subjected to the determination of phosphate content and degradation by phosphomonoesterases. As shown in Table I (Experiment 2), two moles of phosphate were contained per mole of Compounds A, B, C and D. All of these phosphates were liberated by the incubation with alkaline phosphatase. By 3'-nucleotidase almost no liberation of phosphate from Compounds A, B and C was observed. These results suggest that two moles of monoester-bond phosphates are located at 2' and 5' position of the ribose; Compound A or B is nicotinamide riboside 2',5'-diphosphate and Compound C is adenosine 2',5'-diphosphate. From Compound D, 3'-phosphate was liberated; this fact suggests that this compound is the mixture of adenosine 2',5'- and 3',5'-diphosphate.

These results indicate that the nicotinamide ribose moiety of NADP was phosphorylated to give NADP phosphate (Compound V). The NADP analog phosphate (Compound VI) was, however, formed by the phosphorylation of adenosine moiety, but not of the nicotinamide moiety, in NADP analog. Furthermore, it was suggested that Compound VI may be the mixture of the compounds in which the phosphoryl group is located at 2' or 3' position of the

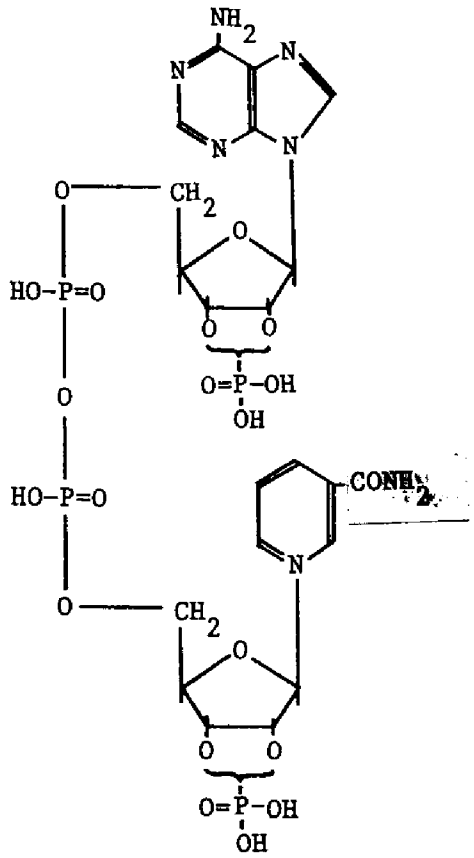

Fig. 8. Structure of Compound V or VI (NAD diphosphate)

ribose of adenosine. Consequently, the chemical structures of NADP diphosphate and NADP analog phosphate may be similar each other as presented in Fig. 8. It is also pressumed that Compound II which was formed besides NADP and NADP analog by the phosphorylation of NAD (see Section 1) may be identical with these derivatives.

SUMMARY

NADP and NADP analog were phosphorylated to NAD diphosphate, and NADP analog phosphate, respectively, by an enzyme preparation of *Proteus mirabilis* (IFO 3849). The degradation products from NAD-diphosphate and NADP analog phosphate by the snake venom nucleotide pyrophosphatase were identical with nicotinamide riboside diphosphate and adenosine 2'(3'), 5'-diphosphate.

Chapter II. Reaction of an NADP Analog and NAD Diphosphate with Dehydrogenases

INTRODUCTION

In the previous chapter, it was shown that under acidic conditions NAD was phosphorylated enzymatically to NADP ($NR\overset{P}{P}PRA$)* and the NADP analog ($N\overset{P}{R}PPRA$) which contains a monoester phosphate group at the 2' or 3' position of nicotinamide riboside. NADP and the NADP analog were also phosphorylated to form NAD diphosphate ($N\overset{P}{R}PP\overset{P}{R}A$).

Kaplan et al.[89-91] reported that NADase obtained from pig brain was capable of catalyzing and exchange reaction between various substituted pyridine compounds and NAD to form analogs of NAD, and that some of them showed coenzymic activity toward dehydrogenases. Shuster and Kaplan[87] showed that the 2' phosphate of NADP was interconverted to its 3' position and that 3' NADP formed thus was reduced by some dehydrogenase systems.

In this chapter experiments with the NADP analog and NAD diphosphate were studied in various dehydrogenase system in order to determine their ability to replace NAD or NADP as co-

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* N, A, R and P designate nicotinamide, adenine, ribose and phosphate moieties, respectively; NADP = $NR\overset{P}{P}PRA$; NADP analog = $N\overset{P}{R}PPRA$; NAD diphosphate = $N\overset{P}{R}PP\overset{P}{R}A$.

enzymes. The inhibitng effect of two new NAD derivatives on pyridine coenzymes was also discussed.

MATERIALS AND METHODS

Preparation of NADP analog and NAD diphosphate. The NADP analog and NAD diphosphate were obtained according to the method described in the previous chapter using ammonium sulfate 30 - 80 % saturated fraction of cell-free extract from *Proteus mirabilis* (IFO 3849). Reduced NADP analog was prepared according to the method of Shuster and Kaplan[87] employing sodium hydrosulfite as the reducing agent. The same amount of NAD was also reduced in this manner to use as a control.

Determination of coenzyme activity. The reduction or oxidation of each of coenzymes and their derivatives was followed by change in absorbancy at 340 mμ on Shimazu multipurpose spectrophotometer model 50L. The amount of reduced or oxidized coenzymes after initial 3 min reaction were calculated from the molecular extinction coefficient of 18.0×10^6 or 6.22×10^6 cm^2 for oxidized or reduced form respectively.

Determination of inhibiting activity. Inhibiting activity of the NADP analog and NAD diphosphate on dehydrogenase was estimated by measuring the initial rate of reduction or oxidation of coenzymes in the presence or absence of

the coenzyme derivatives.

Enzymes. Glucose 6-phosphate dehydrogenase (G6PDH) of yeast, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) of rabbit muscle, isocitrate dehydrogenase (ICDH) of pig heart, malate dehydrogenase (MDH) of pig heart and alcohol dehydrogenase (ADH) of yeast were obtained from C. F. Boehringer and Söhne GmbH. Lactate dehydrogenase (LDH) of pig heart and UDPG dehydrogenase (UDPGDH) of bovine liver were the products of Sigma Chemical Co. NADP-dependent isocitrate dehydrogenase was prepared from baker's yeast according to the method of Kornberg[58]; glucose 6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (IFO 3076) according to DeMoss[92]; glutamate dehydrogenase (GlDH) from *Corynebacterium glutamicum* (ATCC 13059), according to Oshima *et al.*[93]; UDPG dehydrogenase from bovine liver, according to Wilson.[94]

RESULTS

Reaction of the NADP analog with NAD-dependent dehydrogenases.

The NADP analog was not reduced in the dehydrogenase systems, which required NAD as coenzyme, such as alcohol dehydrogenase of yeast, lactate dehydrogenase of pig heart and UDPG dehydrogenase of bovine liver. The initial rate of NAD reduc-

TABLE I. REACTION OF THE NADP ANALOG WITH

Enzyme	Origin	Coenzyme activity test: Experiment No.	Addition	Addition ($\times 10^{-4}$M)	Reduced* ($\times 10^{-4}$M)
ADH	Yeast	(1)	NAD	1.2	0.97
			Analog	1	0
				5	0
LDH	Pig heart	(3)	NAD	1	0.77
			Analog	1	0
UDPGDH	Bovine liver	(5)	NAD	1	0.23
			Analog	1	0
				5	0
GAPDH	Rat liver	(7)	NAD	1	1.0
			Analog	1	0.32
					Oxidized
MDH	Pig heart	(8)	$NADH_2$	1	0.22
			$AnalogH_2$	1.2	0

* during 3 min ** per initial 1 min

Each reaction was carried out in the following systems in a total volume of 3 ml at room temperature.

ADH: ethanol, 20 µmoles; pyrophosphate buffer (pH 8.8), 160 µmoles;

plus { (1) NAD, 0.36 µmole or analog, 0.3 or 1.5 µmoles; ADH, 60 µg.
(2) NAD, 0.36 µmole; analog, 0.3 or 1.5 µmoles; ADH, 1.2 µg.

LDH: Na-lactate, 100 µmoles; glycine buffer (pH 10.0), 200 µmoles;

plus { (3) NAD or analog, 0.3 µmole; LDH, 100 µg.
(4) NAD, 0.3 µmole; analog, 1.5 µmoles; LDH, 0.5 µg.

UDPGDH: UDPG, 2.4 µmoles; glycine buffer (pH 8.6), 300 µmoles;

plus { (5) NAD, 0.3 µmole of analog, 0.3 or 1.4 µmoles; UDPGDH, 530 µg.
(6) NAD, 0.3 µmole; analog, 0.3 or 1.5 µmoles; UDPGDH, 530 µg

NAD-DEPENDENT DEHYDROGENASES.

Experiment No.	Inhibitng activity test: Plus analog ($\times 10^{-4}$M)	NAD reduced** ($\times 10^{-4}$M)
(2)	0	0.065
	1	0.063
	5	0.070
(4)	0	0.025
	5	0.026
(6)	0	0.093
	1	0.093
	5	0.092
		Oxidized
(9)	0	0.077
	1.2	0.072

GAPDH: (7) glyceraldehyde 3-phosphate, 1.15 µmoles; Tris-HCl buffer (pH 8.5), 300 µmoles; Na-arsenate, 51 µmoles; Na-fluoride, 60 µmoles; L-cysteine, 10 µmoles; NAD or analog, 0.3 µmole; GAPDH, 100 µg.

MDH: oxaloacetate, 2.5 µmoles; phosphate buffer (pH 7.5), 300 µmoles;

plus { (8) $NADH_2$, 0.12 µmole or analogH_2, 0.15 µmole; MDH, 5 µg.
plus { (9) $NADH_2$, 0.12 µmole; analogH_2, 0.15 µmole; MDH, 5 µg.

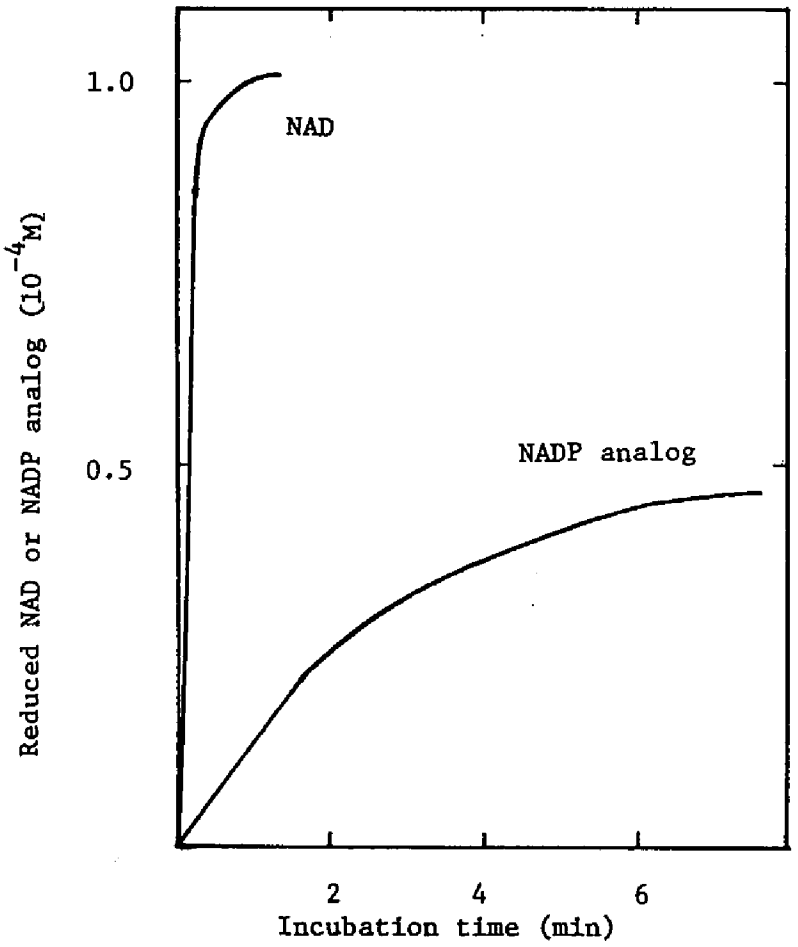

Fig. 1. Reaction of the NADP Analog with Glyceraldehyde 3-phosphate Dehydrogenase.

The reaction system was the same as in reaction (7) of Table I.

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tion by these dehydrogenases was not influenced by the addition of the same or 5-fold amounts of the NADP analog (Table I). Only with high concentration (100 μg per 3 ml) of rat liver

TABLE II. EFFECT OF PREINCUBATION ON THE REDUCTION OF THE NADP ANALOG BY GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE.

	NADP analog reduced after 6 min ($\times 10^{-4}$M)
Control (No preincubation)*1)	0.31
Preincubation system*2)	0.31

*1) The reaction mixture (3 ml) contained 1.2 μmoles of glyceraldehyde 3-phosphate, 300 μmoles of Tris-HCl buffer (pH 8.5), 51 μmoles of Na-arsenate, 60 μmoles of Na-fluoride, 20 μmoles of L-cystein, 0.3 μmole of the NADP analog and 100 μg of GAPDH. The reaction was carried out at room temperature for 6 min.
*2) The NADP analog was incubated with the enzyme preparation in the reaction system shown above without glyceraldehyde 3-phosphate. After 15 min, the substrate was added to the system and an increase of absorbancy at 340 mμ during successive 6 min incubation was measured.

glyceraldehyde 3-phosphate dehydrogenase, reduction of the NADP analog was observed as shown in Fig. 1, whereas with low enzyme concentration (2 μg addition), the analog showed no detectable activity during the reaction intervals tested. This enzyme preparation showed no activity of degrading NADP analog to NAD and an increase of absorbancy at 340 mμ was caused by the net reduction of the NADP analog. This was demonstrated from the fact that the amount of reduced NADP analog in the control system was equal to that in the preincubation system as shown in

TABLE III. EFFECT OF pH ON THE REDUCTION OF THE NADP ANALOG BY GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE

	pH		
	4.0*	7.2**	8.5**
NADP analog reduced after 3 min (x 10^{-4}M)	0	0.33	0.45

* acetate buffer ** Tris-HCl buffer

The reaction system was the same as in the reaction (7) of Table I with each buffer solution.

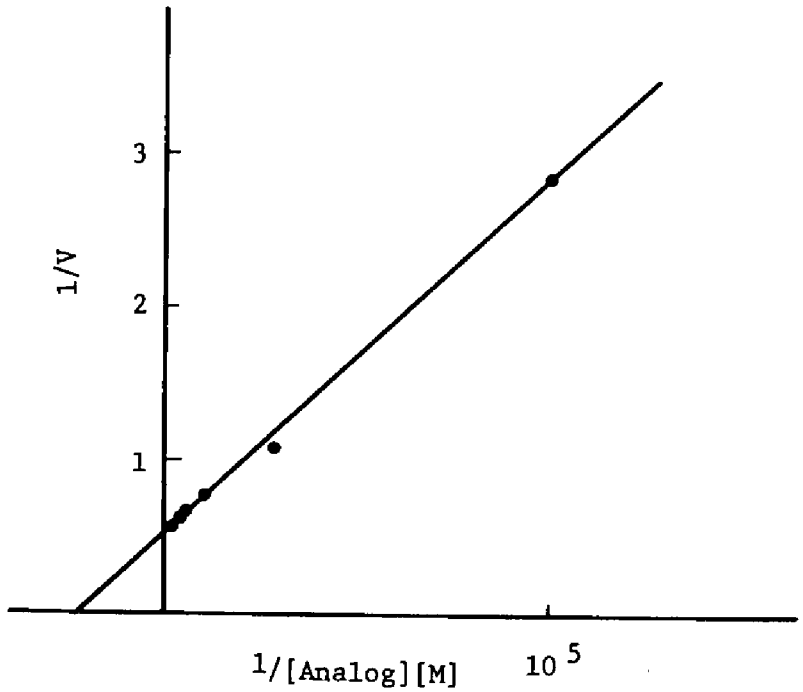

Fig. 2. Effect of concentration of the NADP Analog on Glyceraldehyde 3-phosphate dehydrogenase activity.

The reaction system was the same as in reaction (7) of Table I with each concentration of the NADP analog (10^{-5} - 10^{-3} M).

Table II. The alkaline condition was optimal for the reduction of the NADP analog (Table III). The effect of the analog concentration on the activity of this enzyme was investigated and Km value was calculated from Lineweaver-Burk plot as shown in Fig. 2. The Km value for the NADP analog was found to be 4.4 x 10^{-5}M and was nearly equal to that for NAD (6.7 x 10^{-5}M), which was obtained under low enzyme concentration systems.

Reoxidation of the chemically reduced analog by malate dehydrogenase was tested using oxaloacetate as a substrate. The analog was found to be ineffective in this system and had no inhibiting activity on the oxidation of $NADH_2$.

Reaction of the NADP analog with NADP-dependent dehydrogenases.

The NADP analog was not reduced by NADP-dependent dehydrogenases such as glucose 6-phosphate dehydrogenases of yeast and *Leuconostoc mesenteroides*, isocitrate dehydrogenases of pig heart and baker's yeast and glutamate dehydrogenase of *Corynebacterium glutamicum* as shown in Table IV. The reduction of NADP by these enzymes was not inhibited by the NADP analog.

Reaction of NAD diphosphate with NAD- or NADP-dependent dehydrogenases.

TABLE IV. REACTION OF THE NADP ANALOG WITH

Enzyme	Origin	Coenzyme activity test: Experiment No.	Addition ($\times 10^{-4}$M)		Reduced* ($\times 10^{-4}$M)
G6PDH	Yeast	(1)	NADP	1.2	0.97
			Analog	1	0
				5	0
	L. mesenteroides	(3)	NADP	1.2	0.20
			Analog	1	0
				5	0
ICDH	Pig heart	(5)	NADP	0.86	0.58
			Analog	0.46	0
				2.9	0
	Yeast	(7)	NADP	1	0.44
			Analog	1	0
				3.3	0
GlDH	*C. glutamicum*	(9)	NADP	1	0.33
			Analog	1	0
				5	0

* during 3 min ** per initial 1 min

Each reaction was carried out in the following systems in a total volume of 3 ml at room temperature.

G6PDH (yeast): glucose 6-phosphate, 5 μmoles; Tris-HCl buffer (pH 7.4), 300 μmoles; $MgSO_4$, 10 μmoles;

plus { (1) NADP, 0.36 μmole or analog, 0.3 or 1.5 μmoles; G6PDH, 20 μg.
(2) NADP, 0.36μomol; analog, 0.3 or 1.5 μmoles; G6PDH, 1 μg.

G6PDH (*L.mesenteroides*) :glucose 6-phosphate, 5 μmoles; Tris-HCl buffer(pH 7.4), 300 μmoles; $MgSO_4$, 10 μmoles;

Plus { (3) NADP, 0.35 μmole or analog, 0.3 or 1.5 μmoles; G6PDH, 1.7 mg.
(4) NADP, 0.35 μmoles; analog, 0.3 or 1.5 μmoles; G6PDH, 1.7 mg.

NADP-DEPENDENT DEHYDROGENASES

Inhibiting activity test		
Experiment No.	Plus analog ($\times 10^{-4}$M)	NADP reduced** ($\times 10^{-4}$M)
(2)	0	0.065
	1	0.063
	5	0.070
(4)	0	0.065
	1	0.057
	5	0.061
(6)	0	0.21
	0.46	0.20
	2.9	0.20
(8)	0	0.22
	1	0.22
	3.3	0.22
(10)	0	0.12
	1	0.12
	5	0.13

ICDH (pig heart): *dl*-isocitrate, 0.6 µmole; Tris-HCl buffer (pH 7.2), 75 µmoles; $MnSO_4$, 10 µmoles;

plus { (5) NADP, 0.25 µmole or analog, 0.14 or 0.87 µmole; ICDH, 1.2 mg.
plus { (6) NADP, 0.25 µmole; analog, 0.14 or 0.87 µmole; ICDH, 1.2 mg.

ICDH (yeast): *dl*-isocitrate, 016 µmole; Tris-HCl buffer (pH 7.2), 75 µmoles; $MgSO_4$, 5 µmoles;

plus { (7) NADP, 0.3 or analog, 0.3 or 1.0 µmole; ICDH, 1.2 mg.
plus { (8) NADP, 0.3 µmole; analog, 0.3 or 1.0 µmole; ICDH, 1.2 mg.

GlDH: Na-glutamate, 100 µmoles; phosphate buffer (pH 8.5), 600 µmoles;

plus { (9) NADP, 0.3 µmole or analog, 0.3 or 1.5 µmoles; GlDH, 0.3 mg.
plus { (10) NADP, 0.3 µmole; analog, 0.3 or 1.5 µmoles; GlDH, 0.3 mg.

TABLE V. REACTION OF NAD DIPHOSPHATE

Enzyme	Origin	Coenzyme activity test			
		Experiment No.	Addition	($\times 10^{-4}$M)	Reduced* ($\times 10^{-4}$M)
G6PDH	Yeast	(1)	NADP	1	0.96
			NAD-PP	1	0
				10	0
ICDH	Pig heart	(3)	NADP	1	0.80
			NAD-PP	1	0
				10	0
ADH	Yeast	(5)	NADP	1	0.98
			NAD-PP	1	0
				10	0
LDH	Pig heart	(7)	NAD	1	0.91
			NAD-PP	1	0
				10	0
UDPGDH	Bovine liver	(9)	NAD	1	0.53
			NAD-PP	1	0

* during 3 min ** NAD diphosphate *** per initial 1 min

Each reaction was carried out in the following systems in a total volume of 0.6 ml at 28 C.

G6PDH: glucose 6-phosphate, 1μmole; Tris-HCl buffer (pH 7.4), 90 μmoles; $MgSO_4$, 2 μmoles;

plus { (1) NADP, 0.06 μmole or NAD-PP, 0.06 or 0.6 μmole; G6PDH, 8 μg.
(2) NADP, 0.06 μmole; NAD-PP, 0.06 or 0.6 μmole; G6PDH, 0.2 μg.

ICDH: *dl*-isocitrate, 0.175 μmole; Tris-HCl buffer (pH 7.2), 6.25 μmoles; $MnSO_4$, 2.5 μmoles;

plus { (3) NADP, 0.06 μmole. or NAD-PP, 0.06 or 0.6 μmole; ICDH, 0.4 mg.
(4) NADP, 0.06 μmole; NAD-PP, 0.06 or 0.6 μmole; ICDH, 0.4 mg.

WITH DEHYDROGENASES

inhibiting activity test		
Experiment No.	Plus NAD-PP**	Coenzyme reduced*** ($\times 10^{-4}$M)
(2)	0	0.39
	1	0.39
	10	0.39
(4)	0	0.39
	1	0.40
	10	0.36
(6)	0	0.34
	1	0.30
	10	0.31
(8)	0	0.23
	1	0.26
	10	0.24
(10)	0	0.21
	1	0.21

ADH: ethanol, 40 µmoles; pyrophosphate buffer (pH 8.8), 32 µmoles;

plus { (5) NAD, 0.06 µmole or NAD-PP, 0.06 or 0.6 µmole; ADH, 60 µg.
plus { (6) NAD, 0.06 µmole; NAD-PP, 0.06 or 0.6 µmole; ADH, 1 µg.

LDH: Na-lactate, 25 µmoles; glycine buffer (pH 10.0), 40 µmoles;

plus { (7) NAD, 0.06 µmole or NAD-PP, 0.06 or 0.6 µmole; LDH, 50 µg.
plus { (8) NAD, 0.06 µmole; NAD-PP, 0.06 or 0.6 µmole; LDH, 7 µg.

UDPGDH: UDPG, 2.35µmoles; glycine buffer (pH 8.6), 150 µmoles;

plus { (9) NAD, 0.06 µmole of NAD-PP, 0.06 µmole; UDPGDH, 333 µg.
plus { (10) NAD, 0.06 µmole; NAD-PP, 0.06 µmole; UDPGDH, 333 µg.

NAD diphosphate was not reduced by NADP-dependent dehydrogenases such as glucose 6-phosphate dehydrogenase of yeast and isocitrate dehydrogenase of pig heart. This substance did not also react with NAD-dependent enzymes such as alcohol dehydrogenase of yeast, lactate dehydrogenase of pig heart and UDPG dehydrogenase of bovine liver. The reduction of coenzymes by these dehydrogenases was not inhibited by NAD diphosphate. These results are shown in Table V.

DISCUSSION

The study of the oxidoreduction of NAD derivatives with various dehydrogenases offered some insight into the relation between chemical structure of pyridine coenzymes and their coenzyme activity. It was found in this investigation that the NADP analog (N$\overset{P}{R}$PPRA) and NAD diphosphate (N$\overset{P}{R}$PP$\overset{P}{R}$A) showed no or slight coenzyme activity for dehydrogenases tested. By the introduction of monoester-phosphoryl group on the nicotinamide riboside or on both nicotinamide riboside and adenosine, NAD lost almost its coenzyme activity for NAD-dependent dehydrogenases. Only glyceraldehyde 3-phosphate dehydrogenase system reduced slightly the NADP analog. However, it was suggested that the anaolg has a lower affinity toward this enzyme than NAD. It appears from these results that changes of the position

of phosphoryl group in pyridine coenzymes bring about pronounced effects on coenzyme activity. The phosphoryl group in adenosine moiety may be essential for NADP specific dehydrogenase activity. Shuster and Kaplan[87] have shown that 3'NADP in which the phosphoryl group is located at the 3' position of adenosine moiety react with enzymes capable of functioning with both of NAD and NADP, but not with enzymes which are strictly specific for one of these coenzymes. This observation seems to support the results mentioned in the present paper.

The relationship between the ring structure and the coenzyme activity has been discussed by Kaplan and the associates[36,91] and it was shown that the nicotinamide group was not essential for NAD activity. In the series of pyridine-substituted NAD analogs, the pyridine-3-aldehyde analog is not only inactive for the triosephosphate dehydrogenase, but also a marked inhibitor toward this enzyme system. However, the NADP analog and NAD-diphosphate showed no inhibiting activity toward glyceraldehyde 3-phosphate dehydrogenase.

SUMMARY

The NADP analog and NAD diphosphate were tested for the coenzyme or inhibiting activity toward various dehydrogenases. Little or no activity of these NAD derivatives was observed for most of dehydrogenases tested. Only glyceraldehyde 3-phosphate dehydrogenase reduced the NADP analog under the high enzyme concentration system. These NAD derivatives showed no inhibiting effect toward the reduction or oxidation of pyridine coenzymes.

Chapter III. Distribution and Properties of NAD Phosphorylating Reaction

INTRODUCTION

In Chapter I it was shown that NAD was enzymatically phosphorylated to NADP and an NADP analog by a new phosphotransferring system in acidic conditions and that NAD kinase did not participate in this transferring reaction. Phosphorylating products of other pyridine nucleotides were also obtained with the same system.

It has been shown that alcohols, sugars and sugar derivatives were phosphorylated by phosphatase[53,95,96] or phosphotransferase.[50,97,98] Phosphorylation of pyridine nucleotides was considered to be catalyzed by these enzymes.

In this chapter, distribution of a new NAD phosphorylating system, designated tentatively as phosphotransferase, and NAD kinase was investigated in a variety of microorganisms. General properties of phosphotransferase were also discussed employing the partially purified enzyme preparation obtained from *Proteus mirabilis*.

MATERIALS AND METHODS

Microorganisms and enzyme preparation. All the micro-

organisms used in this experiment were the strains preserved in the Laboratory of Applied Microbiology, Department of Agricultural Chemistry, Kyoto University.

Bacteria and actinomycetes were grown in a liquid medium of the following composition: glucose, 2.0%; peptone, 1.0%; yeast extract, 0.5%; $(NH_4)_2SO_4$, 0.3%; NaCl, 0.2%; K_2HPO_4, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.05%. Final pH was adjusted to 7.0. Culture was carried out in 500 ml of the medium in 2L shaking flask with a reciprocal shaker for 24 to 48 hr at 28°C. *Acetobacter suboxidans* was grown in a medium of the following composition: glycerol, 5.0%; yeast extract, 0.5%; KH_2PO_4, 0.25%; $CaCO_3$, 2.0%. Final pH was adjusted to 6.0. The culture was carried out in 500 ml of the medium in 2L shaking flask with a reciprocal shaker for 48 hr at 28°C. Clostridia was grown in a medium of the following composition: glucose, 3.0%; peptone, 0.5%; K_2HPO_4, 0.3%; $MgSO_4 \cdot 7H_2O$, 0.02%; $MnSO_4 \cdot xH_2O$, 0.001%; NaCl, 0.001%; yeast extract, 0.1%. Final pH was adjusted to 7.0. One percent of $CaCO_3$ was added to the medium before inoculation. The culture was carried out in 5L of the medium in 5L Ehrenmeyer flask for 36 hr at 28°C stationary. Lactic acid bacteria were grown in a medium of the following composition: glucose, 1.1%; Na-acetate, 1.0%; peptone, 0.8%; yeast extract, 0.8%; KH_2PO_4, 0.03%; K_2HPO_4, 0.025%; $MgSO_4 \cdot 7H_2O$, 0.01%; $MnSO_4 \cdot xH_2O$, 0.01%; $FeSO_4 \cdot 7H_2O$, 0.0005%. Thirty per cent

(v/v) of hot water extract of tomato puree was added to the medium and pH was adjusted to 7.0. The culture was carried out in 2L of the medium in 3L Ehrenmeyer flask for 8 hr at 28°C stationary.

Cells were harvested by centrifugation, washed twice with 0.85% NaCl solution and suspended in 0.05 M Tris-HCl buffer (pH 7.0). The cell suspension was treated with Kaijo Denki ultrasonic oscilator at 20 Hz for 10 min. Cell debris were removed by centrifugation and the supernatant cell-free extract was dialyzed against deionized water at 4°C for 24 hr. This dialyzed solution was employed as the enzyme preparation.

Molds were grown in a medium of the following composition: glucose, 5.0%; peptone, 0.5%; yeast extract, 0.2%; KH_2PO_4, 0.2%; $(NH_4)_2SO_4$, 0.2%; $MgSO_4$ $7H_2O$, 0.1%. Final pH was adjusted to 6.0. The culture was carried out in 500 ml of the medium in 2L shaking flask for 24 to 48 hr at 28°C with reciprocal shaker. Mycelia were collected by filtration and dried with electric fan. Fine powder of crushed mycelia were used as the enzyme preparation.

Partial purification of phosphotransferring enzyme of Proteus mirabilis. All operations were performed in the cold.

Procedure 1. Results of a typical experiment are summarized in Table I.

TABLE I. PARTIAL PURIFICATION OF PHOSPHOTRANSFERASE

Step	Procedure	Total protein mg	Total activity		
			Phosphatase		Phosphotransferase
			without NAD	with NAD	
1	Cell-free extract	1755	4080	3190	1330
2	Protamine treatment	610	4020	2900	1070
3	Polyethylene glycol treatment	285	2300	1760	715
4	DEAE-Sephadex	32.5	2220	1705	595

The reaction was carried out with Mixture 3.

Step 1: Cells (2.6g as dried weight) suspended in 95 ml of 0.01 M Tris-HCl buffer (pH 7.0) were disrupted with Kaijo Denki ultrasonic oscillator at 20 Hz for 15 min. Cell debris were removed by centrifugation at 14,000 x g for 40 min and the supernatant solution (75 ml) was obtained.

Step 2: To this solution 10.2 ml of 5% protamine solution (adjusted to pH 7.0) was added with stirring. About 3 mg of protamine sulfate was added for every 10 mg of protein. After stirring for 1 hr, the precipitates were removed by centrifugation at 14,000 x g for 15 min and the supernatant solution (74 ml) was obtained.

AND PHOSPHATASE

Specific activity			Transferring ratio*
Phosphatase		Phosphotrans-ferase	
without NAD	with NAD		
2.3	1.8	0.8	0.42
6.6	4.8	1.8	0.37
8.1	6.2	2.5	0.40
68.2	52.6	18.3	0.35

* Molar ratio NADP plus NADP analog formed to *p*-NP liberated

Step 3: The solution was brought to a 0.05 M magnesium chloride concentration by the addition of 3.4 ml of molar magnesium chloride. To the solution, 15 g of solid polyethylene glycol 6000 was added gradually so as to bring about 20 % (w/v) of the polymer. The precipitates were collected by centrifugation at 14,000 x g for 15 min and suspended in 15 ml of 0.05 M Tris-HCl buffer (pH 7.0). The supernatant solution was obtained by centrifugation at 14,000 x g for 10 min after continuous stirring overnight.

Step 4: This solution (15.5 ml) was applied to a DEAE-Sephadex column (1.2 cm x 35 cm) previously equilibrated with

0.05 M Tris-HCl buffer (pH 7.0). After the column was washed with 0.05 M Tris-HCl buffer (pH 7.0), protein was eluted with the same buffer solution containing 0.05, 0.1 and 0.15 M of NaCl in a stepwise manner. Fractions of 10 ml were collected and assayed for protein, phosphotransferase and phosphatase activities. Elution pattern is shown in Fig. 1. Phosphotransferase and phosphatase activities were eluted by 0.1 M NaCl. The fractions which showed the activities (fraction 53 to 73) were conbined and, after dialysis against deionized water, stored in the frozen state.

Procedure 2. Preparation of Step 2 of Procedure 1 was applied to a DEAE-Sephadex column previously equilibrated with 0.05 M Tris-HCl buffer and protein was eluted by increasing NaCl concentration in a stepwise manner as in Step 4 of Procedure 1. Phosphatase and phosphotransferase were eluted by the addition of 0.1 M NaCl. Fractions which showed the enzyme activities were conbined and polyethylene glycol was added to bring about 20% (w/v) solution of the polymer in the presence of 0.05 M of magnesium chloride. The protein which was adsorbed to the precipitates was extracted with 0.05 M Tris-HCl buffer (pH 7.0) and the extract was dialyzed against deionized water.

Enzyme assay. Reaction mixture 1: In the investigation on distribution of phosphotransferase activity, each assay mixture

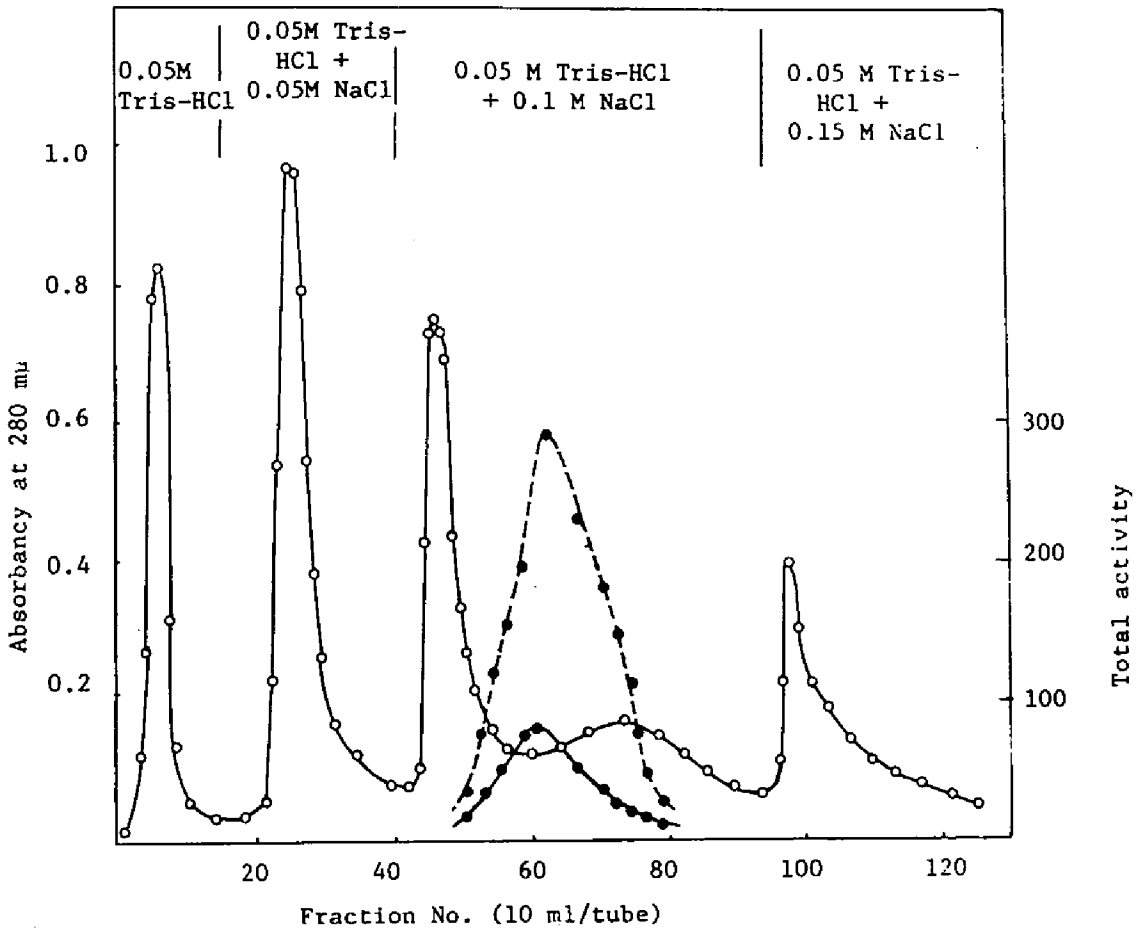

Fig. 1. Chromatography of Phosphotransferase on DEAE-Sephadex Column.

o——o Protein

●----● Phosphatase activity

●——● Phosphotransferase activity

contained 10 μmoles of NAD, 40 μmoles of *p*-NPP, 20 μmoles of nicotinamide, 1 μmole of zinc sulfate, 300 μmoles of acetate buffer (pH 4.0) and 5 mg of enzyme protein of bacteria or 10 mg of dried mycelia of molds in a total volume of 1 ml. Unless otherwise mentioned, reaction was carried out at 37°C for 2 or 6 hr with bacteria or mold enzyme, respectively.

Reaction mixture 2: In the investigation of properteis of the partially purified enzyme preparations, each assay mixture contained 40 μmoles of phosphoryl donor, 10 μmoles of phosphoryl acceptor, 1μmole of cupper sulfate, 300 μmoles of acetate buffer (pH 4.0) and enzymes in a total volume of 1 ml.

Reaction mixture 3: Preparations obtained in each purification step were assayed for both transferase and phosphatase activities with the assay mixture containing 40 μmoles of *p*-NPP, 10 μmoles of NAD, 1 μmole of cupper sulfate; 300 μ moles of acetate buffer (pH 4.0) and enzyme protein in a total volume of 1 ml. Incubation was carried out for 30 min at 37°C. The amount of enzyme to be added was adjusted to be within the linear range of product formation.

Fractions eluted from the column of DEAE-Sephadex were assayed also for both transferase and phosphatase activities. In the assay of phosphotransferase activity inosine was added as a phosphoryl acceptor.

Portions of 0.1 to 0.5 ml of the reaction mixture (Reaction mixture 1, 2 and 3) were taken for the estimation of *p*-NP according to the method of Omori.[99] The nucleotides formed in 0.05 ml of the reaction mixture were separated by paper chromatography in a solvent system consisted of isobutyric acid and 0.5 N ammonium hydroxide (10 : 6, v/v),[55] followed by spectrophotometry in the ultraviolet for nucleotides and by fluorometry with methyl ethyl ketone for pyridine nucleotides mentioned in Chapter I.

Reaction mixture 4: In the investigation on distribution of NAD kinase, each assay mixture contained 2.5 μmoles of NAD, 10 μmoles of ATP, 15 μmoles of $MgSO_4$, 100 μmoles of potassium phosphate buffer (pH 7.0) and 1 mg of enzyme protein.

NADP formed in the reaction mixture (Reaction mixture 4) was determined enzymatically employing a assay mixture containing 5 μmoles of *dl*-isocitrate, 75 μmoles of Tris-HCl buffer (pH 7.2), 5 μmoles of $MgSO_4$, 1 mg of isocitrate dehydrogenase and suitable aliquot of the supernatant solution of Reaction mixture 4 which was adjusted to contain within 0.5 μmole of NADP in a total volume of 1 ml. An increase of absorbancy at 340 mμ was measured with 1.0 cm light path on Hitachi-Perkin-Elmer spectrophotometer model 139. The reaction mixture was incubated about for 30 min at room temperature until absorbancy at 340 mμ reached a maximum. NADP reduced was calculated from the molar extinction coefficient

of 6.22×10^6 cm^2.

Expression of enzyme activity. One unit of phosphotransferase and phosphotase activities was defined as the amount which formed 1 μmole of phosphorylation product and *p*-NP, respectively. Specific activity was expressed as units per mg of protein. Protein was estimated according to the method of Lowry[70] and to the method of Warburg and Christian[71] for the preparation of purification steps 3 and 4.

Chemicals and enzyme. *p*-NPP was the gift from Ajinomoto Co Ltd. NADP-specific isocitrate dehydrogenase of pig heart was the product of Sigma Chemical Co. Other chemicals were obtained from commercial sources.

RESULTS

I. Distribution of NAD phosphorylating activity

NAD phosphorylating activities were searched for various strains of microorganisms. Results are shown in Table II.

NAD kinase activity was distributed rather widely in bacteria belonging to genera *Escherichia, Aerobacter, Proteus, Bacillus, Aerobacterium, Micrococcus, Bacterium, Acetobacter* and *Pseudomonas*. However, slight or no activity was observed under the conditions mentioned in the Methods and Materials in the strains belonging to genera *Serratia, Flavobacterium, Corynebacterium, Lactobacillus,*

TABLE II. DISTRIBUTION OF NAD PHOSPHORYLATING ACTIVITIES IN MICROORGANISMS

Strain	NAD kinase NADP, μmole/ml	Phosphotransferase NADP + Analog, μmoles/ml
Escherichia coli Crooks	0.11	0.02
Escherichia coli 2bT	0.16	0.01
Escherichia coli K12 IFO 3208	0.14	0.05
Escherichia freundii S-96	0.21	0.03
Escherichia intermedia A-21	0.25	0.06
Aerobacter aerogenes IFO 3320	0.19	0.24
Aerobacter cloacae	0.18	0.06
Serratia marcescence IFO 3167	0.18	0.13
Proteus vulgalis IFO 3045	0.21	0.23
Proteus mirabilis IFO 3849	0.20	1.37
Proteus morganii IFO 3848	0.29	0.03
Alcaligenes faecalis IAM B-141-1	0.07	0
Alcaligenes viscolactis IAM 1517	0	0
Flavobacterium flavescence IFO 3085	0	0
Flavobacterium arborescens IFO 1100	0.02	0
Bacillus megaterium	0.02	0
Bacillus subtilis IFO 3007	0.08	0
Aerobacterium tumefaciens IAM B-26-1	0.15	0
Aerobacterium radiobacter IAM 1526	0.08	0
Micrococcus urea IAM 1010	0.06	0
Micrococcus flavus IFO 3242	0.04	0.06
Micrococcus glutamicus ATCC 13032	0.28	0
Staphylococcus aureus IFO 3061	0.02	0.16
Sarcina aurentica IFO 3064	0.09	0
Sarcina lutea IAM 1099	0.06	0

(continued)

Corynebacterium sepedonicum IFO 3306	0	0
Corynebacterium equi IAM 1038	0.	0
Arthrobacter simplex IFO 3530	0	0
Brevibacterium ammoniagenes IFO 12071	0.04	0
Bacterium cadaveris IFO 3731	0.29	0.03
Bacterium succinicum	0.07	0.20
Pseudomonas fluorescens IFO 3461	0.14	0
Pseudomonas ovalis IFO 3738	0.05	0
Xanthomonas malcescence IFO 3383	0.05	0
Acetobacter suboxydans IFO 3172	0.10	0
Clostridium kainantoi	0	0
Leuconostoc mesenteroides IFO 3426	0.06	0
Lactobacillus plantarum IFO 3070	0.01	0
Lactobacillus helveticus IFO 3219	0.04	0
Lactobacillus fermenti	0	0
Mycobacterium avium IFO 3154	0.02	0
Nocardia asteroides IFO 3424	0.01	0
Nocardia corallina IFO 3338	0.04	0
Streptomyces coelicolor IFO 3226	0	0.10
Streptomyces aureofaciens IFO 3305	0.01	0.16
Streptomyces aureus IFO 3175	0	0
Streptomyces griseus IFO 3122	0	0
Penicillium notatum	—	0
Aspergillus niger	—	0
Aspergillus flavus	—	0
Aspergillus oryzae	—	0
Rhizopus japonicus	—	0
Mucor javanicus	—	0
Fusarium line	—	0
Oospora viscosa	—	0

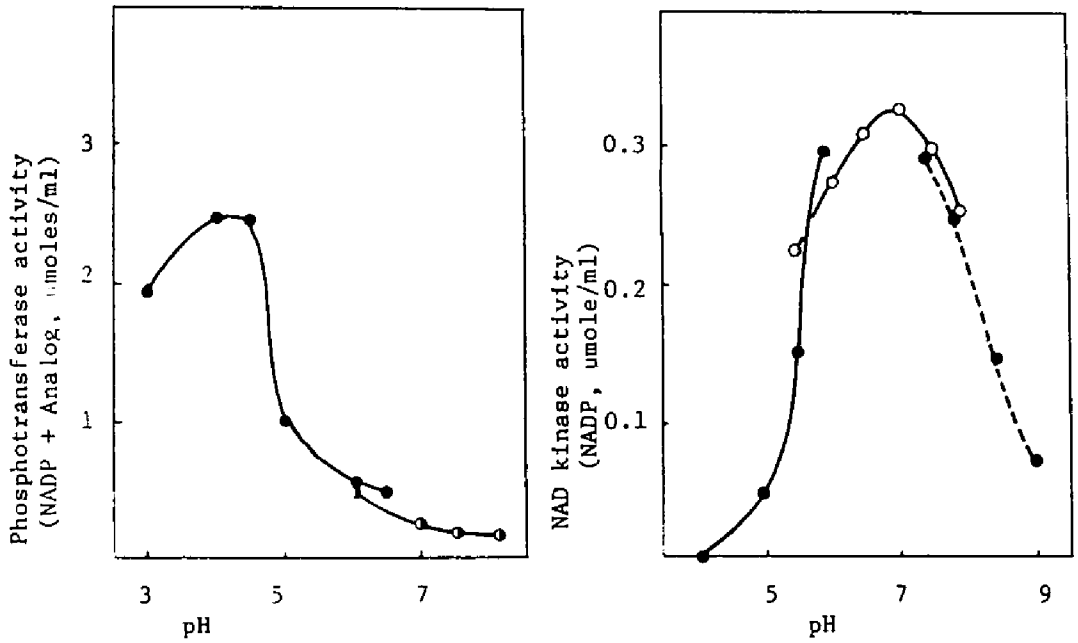

Fig. 2. Effect of pH on Phosphotransferase and NAD Kinase Activities.

●——● Acetate buffer o——o Tris-Maleate buffer
●- - -● Tris-HCl buffer o——o Phosphate buffer

Mycobacterium, *Nocardia* and *Streptomyces*.

On the other hand, phosphotransferase activity was observed only in a few genera such as *Proteus*, *Aerobacter*, *Bacterium* and *Streptomyces*. In bacteria tested, *Proteus mirabilis* showed the highest activity. This organism contained NAD kinase activity besides phosphotransferase activity. Effect of pH on both NAD phosphorylating activities was discussed employing the cell-free extract of the organisms as the enzyme preparation (Fig. 2).

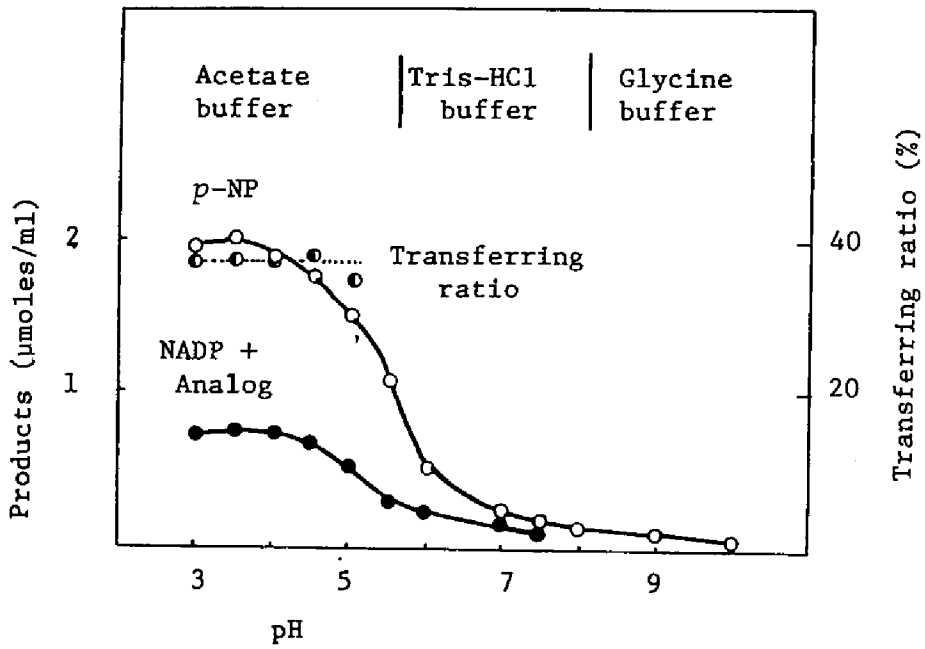

Fig. 3. Effect of pH on Phosphotransferase Activity.

In the condition of pH 4.0, enzyme preparation showed maximal activity of phosphotransferase, whereas no NAD kinase activity was demonstrated. On the other hand, in weak acidic and neutral conditions, either NAD kinase or phosphotransferase were operative.

Strains of molds tested showed no phosphotransferase activity (Table II); only degradation of NAD was observed.

II. Properties of phosphotransferase.

Properties of phosphotransferase activity were investigated using the preparations of Step 4 of Procedure 1 and Procedure 2.

Effect of pH. Effect of pH on the phosphorylation of NAD

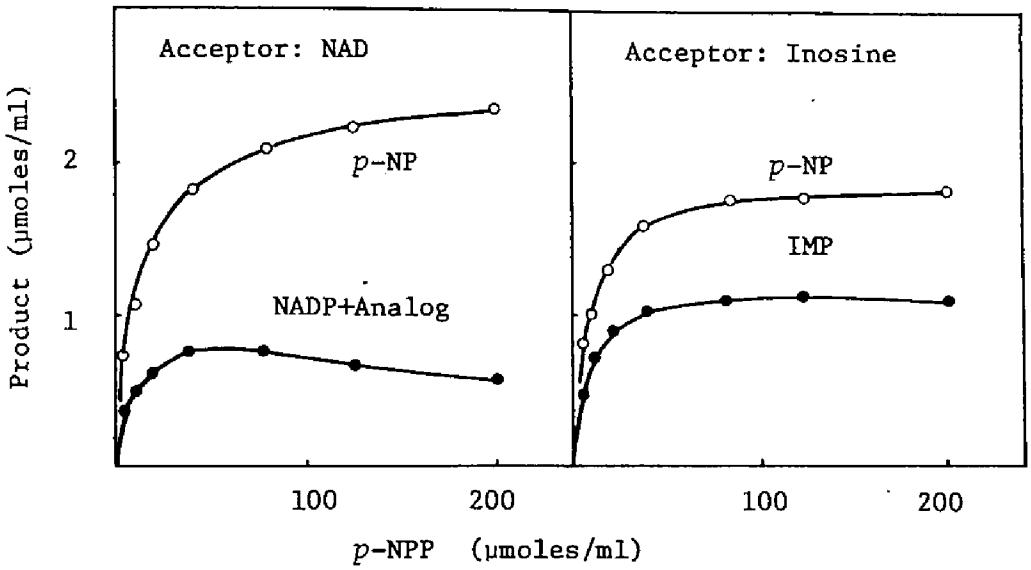

Fig. 4. Effect of *p*-NPP Concentration.

The reaction was carried out in Reaction mixture 2 with various concentrations of *p*-NPP.

and the formation of *p*-NP was examined throughout the range from pH 3 to 10 (Fig. 3). In the pH range in which the enzyme preparation showed high phosphotransferring activity (below pH 5.0), the transferring ratio (see Table I) was almost constant, it being from 0.33 to 0.39. The optimal pH for the phosphorylation of NAD and the formation of *p*-NP in the presence of NAD was found to be about 3.0 to 4.0.

Effect of p-NPP concentration. Effect of *p*-NPP concentration on the phosphotransferase activity was examined using NAD or inosine as phosphoryl acceptor (Fig. 4). The formation of NADP plus NADP analog increased with the concentration of *p*-NPP. In the systems in which *p*-NPP was added more than 40 μmoles per ml

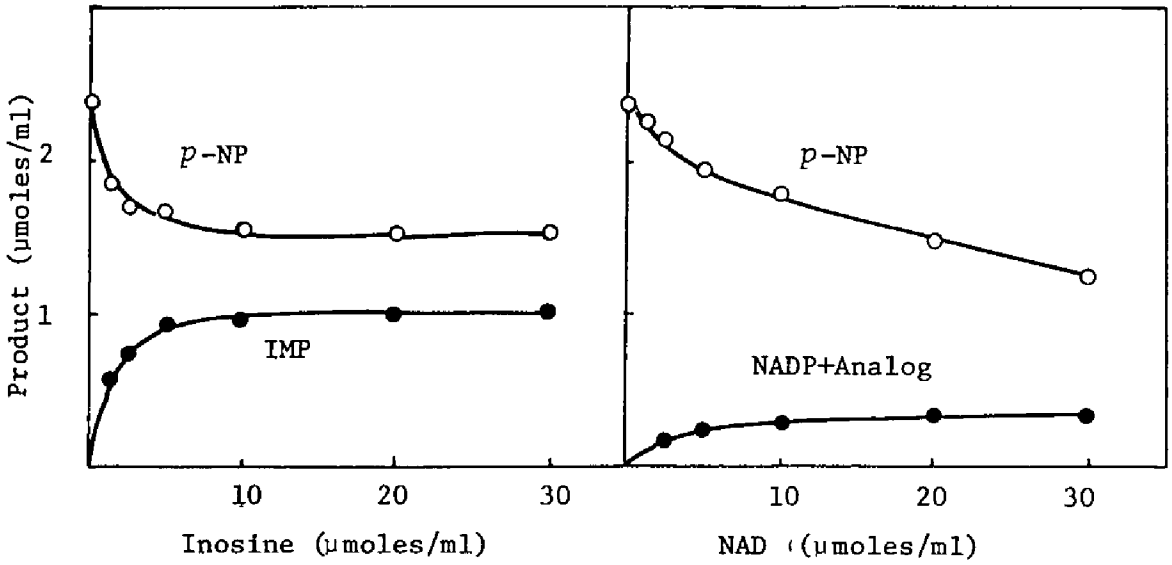

Fig. 5. Effect of Acceptor Concentration.

The reaction was carried out in Reaction mixture 2 with various concentrations of inosine or NAD.

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of the reaction mixture, the phosphorylation of NAD was rather inhibited. Synthesis of IMP reached a maximum under the conditions in which 80 μmoles of *p*-NPP was added per ml of the mixture. Effect of *p*-NPP concentration on phosphatase was also examined. *p*-NPP was hydrolyzed by the enzyme preparation rapidly and the activity reached $v_{maximum}$ with lower concentrations of the substrate than that in the absence of NAD or inosine. Km value for *p*-NPP was calculated to be 4.0×10^{-3} M by the Hofstee plot.

Effect of acceptor concentration. The formation of IMP reached a maximum on the addition of more than 5 μmoles of inosine

TABLE III. DONOR SPECIFICITY

Donor	NADP + Analog (μmoles/ml)
2'-AMP	0.8
3'-AMP	0.2
5'-AMP	0.1
2'(3')-GMP	0.2
5'-GMP	0.2
5'-IMP	0.5
2'(3')-UMP	0.2
5'-UMP	trace
2'(3')-CMP	0.5
5'-CMP	0.1
ATP	trace
p-NPP	1.7
Phenyl-P	0.8
G-6-P	0
None	0

The reaction was carried out with Reaction mixture 2 using the preparation of Procedure 2.

(Fig. 5). Synthesis of NADP plus NADP analog proceeded maximally on the addition of more than 10 μmoles of NAD. Km values for inosine and NAD were calculated by Lineweaver-Burk plot to be 9.9×10^{-2} M and 4.2×10^{-2} M, respectively.

Donor specificity. Various organic phosphate were tested for the ability to act as phosphoryl donor to NAD. As shown in Table III, *p*-NPP was the most excellent donor. Various nucleotides

TABLE IV. ACCEPTOR SPECIFICITY

Acceptor	Phosphotransferase activity		Phosphatase activity
	Product	µmoles/ml	*p*-NP, µmoles/ml
Adenosine	AMP	0.2	2.1
2'-AMP	AR-diP	trace	0.7
3'-AMP	AR-diP	trace	0.4
5'-AMP	AR-diP	1.6	2.8
Guanosine	GMP	1.7	2.5
2'(3')-GMP	GR-diP	0.5	0.6
5'-GMP	GR-diP	2.9	3.6
Inosine	IMP	1.5	2.2
5'-IMP	HR-diP	1.1	3.3
Uridine	UMP	1.0	1.3
2'(3')-UMP	UR-diP	0.2	0.1
5'-UMP	UR-diP	1.1	1.3
Cytidine	CMP	1.2	1.7
2'(3')-CMP	CR-diP	0.1	0.2
5'-CMP	CR-diP	1.1	1.7
NAD	NADP + Analog	0.8	2.9
None	—	—	3.7

The reaction was carried out with Reaction mixture 2 using the preparation of Procedure 2.

served also as donor. Generally, 2'(or3') isomers were more effective than 5' isomers. In the three isomers of AMP, 3' isomer was the most efficient. Sugar phosphate such as glucose 6-phosphate, 5' isomers of UMP and CMP and ATP were nearly or entirely

TABLE V EFFECT OF METAL IONS

Ion*	Phosphotransferase activity (NADP+Analog µmole/ml)	Phosphatase activity (*p*-NP, µmoles/ml)	
		with NAD	without NAD
Cu^{2+}	0.8	2.3	3.9
Zn^{2+}	0.2	0.7	0.9
Mn^{2+}	0.1	0.5	0.9
Mg^{2+}	0.2	0.7	1.3
Ni^{2+}	0.2	0.9	2.1
Co^{2+}	0.2	0.7	1.2
Fe^{2+}	trace	0.2	0.2
Fe^{3+}	trace	0.1	0.1
Sn^{2+}	0.1	0.1	0.1
Ag^{+}	0.1	0.4	0.5
Ba^{2+}	0.1	0.4	0.4
MoO_4^{2-}	0.1	0.2	0.3
Hg_2^{2+}	0.1	0.3	0.5
EDTA	0.1	0.4	0.1
None	0.1	0.4	0.5

The reaction was carried out with Reaction mixture 2 using the preparation of Step 4 of Procedure 1.

* 10^{-3} M

inactive.

Acceptor specificity. Phosphoryl acceptor specificity was examined using nucleosides and nucleotides as acceptors and *p*-NPP as donor.(Table IV) Generally, nucleosides and nucleotides served as acceptor and in the series of nucleotides, 5' isomers were better acceptor. It was also found that phosphatase activity was inhibited by the addition of each acceptor. Acceptors inactive in phosphoryl transfer were the intense inhibitor for phosphatase activity.

Effect of metal ions. Effect of metal ions and EDTA on phosphotransferase and phosphatase activities was examined. As shown in Table V, Cu^{2+} stimulate both activities of phosphotransferase and phosphatase extensively. Zn^{2+}, Mn^{2+}, Mg^{2+}, Ni^{2+} and Co^{2+}, had slso the stimulating effect, while Fe^{2+} Fe^{3+} Sn^{3+}, MoO_4^{2-} and EDTA suppressed the activities.

DISCUSSION

As indicated in Chapter I, NAD was phosphorylated by the enzyme preparation of *Proteus mirabilis* (IFO 3849) through a new phosphotransferring reaction (designated as phosphotransferase tentatively in this chapter) other than NAD kinase. In this experiment the distribution of two pathways concerning phosphorylation of NAD was investigated.

Phosphorylation of nucleosides[50,96-98] and vitamins[100] by phosphatase or phosphotransferase was reported by several investigators. Mitsugi *et al.*[101] reported the distribution of nucleoside phosphotransferase in microorganisms and classified bacteria into two groups; namely one group synthesized 5'-isomers of nucleotide from nucleoside and the other synthesized 2' (or 3') isomers. Brawerman and Chargaff[62] indicated that phosphotransferase was distributed widely in plant, bacteria, protozoa and vertebrates and that this enzyme activity was an attribute of growing cells. They also indicated that phosphotransferase activity of *Escherichia coli* showed significant change during growth; the phosphorylating ratio increased sharply in the log phase of growth and declined steeply with the decrease of growth. Although biological significance of this phosphorylating mechanism has not been elucidated, this fact suggest that the enzyme may participates in nucleotide synthesis. It is noteworthy that the enzyme showed the most intense activity in the phase of rapid growth, that is in the period of maximal nucleic acid synthesis. Evidence for the actual operation of phosphorylation of NAD through the phosphotransferase besides NAD kinase in physiological conditions has not been provided. However, it may be possible that the phosphorylation of NAD by this enzyme is operative especially in the period of rapid growth to supply NADP. Furthermore, the author has observed that *P.*

mirabilis showed high NAD phosphorylating activity in the early period of growth.

In this experiment partial purification of the phosphotransferase was attempted and it was shown that the phosphotransferase preparation possessed high activity of phosphatase. In the separation of enzymes with DEAE-Sephadex column, phosphotransferase and phosphatase activities were eluted in the same fractions. Furthermore, the enzyme preparation in each purification step showed almost constant phosphorylating ratio (0.35 to 0.42) for the phosphorylation of NAD. These results may indicate that phosphoryl transfer and hydrolysis of *p*-NPP are catalyzed by the same enzyme. Brunngraber and Chargaff[98] separated phosphatase and phosphotransferase from the extract of carrot and indicated that phosphorylation of nucleosides was catalyzed by nucleoside phosphotransferase which exhibited hydrolase activity mainly because phosphoryl transfer was favored over hydrolysis.

Optimal pH for phosphoryl transfer was reported to be 5.0 with the enzymes of carrot and *E. coli*[102] and 6.0 with those of prostate and *E. freundii*. The preparation of *P. mirabilis* showed lower pH value, 4.0, for the phosphorylation of NAD. The fact that in weak acidic or neutral conditions a phosphoryl group was transferred from *p*-NPP or nucleoties to NAD suggest the significance of this reaction in NADP synthesis.

Through the experiments concerning phosphoryl donor and acceptor, it was shown that a phosphate group at 2' (or 3') position of nucleotide transferred easily to nucleosides and nucleotides, and of the series of nucleoties, 5' isomers were phosphorylated more rapidly than other isomers. On the other hand, not only *p*-NPP but also various nucleotides could serve as phosphoryl donor for the phosphorylation of NAD. These results suggest the possibility of the occurrance of NAD phosphorylating system other than NAD kinase in microbial cells.

SUMMARY

Distribution of NAD phosphorylating reactions, phosphorylation through NAD kinase and phosphotransferase, was investigated. NAD kinase activity was distributed rather widely in bacterial, whereas the phosphotransferase activity with *p*-NPP and NAD was limited to a few genera. *Proteus mirabilis* showed strong activity of the phosphotransferase besides NAD kinase activity.

Partial purification of the phosphotransferase was attempted. The enzyme preparation possessed phosphatase activity as well as phosphotransferase activity. Phosphorylation of NAD proceeded maximaly under the conditions below pH 4.0. Cu^{2+} showed stimulating effect on the activity. Besides *p*-NPP and phenylphosphate various nucleotides, especially 2' (or 3') isomers, served as

excellent phosphoryl donor, and various kinds of nucleosides and nucleoties were phosphorylated to form nucleoside monophosphates or nucleoside 5',2'(or 3')-diphosphates.

CONCLUSION

It has been shown that synthesis of NADP, phosphorylation of NAD, is catalyzed by NAD kinase and this enzyme is distributed widely in organisms. In this study, it was demonstrated that NAD was phosphorylated by the enzyme preparation of *Proteus mirabilis* under acidic conditions. Besides *p*-NPP, various nucleotides served as the phosphoryl donor, whereas ATP, a phosphoryl donor of NAD kinase, was ineffective. In this phosphorylating reaction, an NADP analog which showed no coenzyme activity for isocitrate dehydrogenase was formed. After the decomposition of coexisting NADP, this compound was separated and isolated by column chromatography. In the NADP analog, a phosphoryl group incorporated was located at 2' or 3' position of nicotinamide riboside, whereas in NADP a phosphate group was located at 2' position of adenosine moiety. This structural properties was demonstrated by the aid of various enzymic and chemical methods. Nicotinamide riboside 2'(3'),5'-diphosphate was obtained on the degradation of the analog with phosphodiesterase. The position of its phosphate group was decided employing several phosphomonoesterase preparations. Other pyridine nucleotides, NMN and NADP, were phosphorylated to form nicotinamide riboside 2'(3'),5'-diphosphate and NAD diphosphate under the same condition. The latter compounds was also formed by the phosphorylation of the NADP analog.

One of the most important functions of pyridine coenzymes is their participation in biological oxido-reduction systems, that is, in coenzyme activity for dehydrogenases. Pyridine nucleotides obtained in this experiment were tested for the coenzyme activity toward various dehydrogenases. The results obtained in this study suggest the relationship between the possition of phosphate in pyridine nucleotide and coenzyme function. NADP analog was reduced only by glyceraldehyde 3-phosphate dehydrogenase under the condition of high enzyme concentration. NADP analog and NAD diphosphate showed almost no activity for other enzymes which were specific to NAD or NADP, and possibility of antagonistic effect on dehydrogenases was discussed. Rate of reduction of NAD or NADP with dehydrogenases was not affected by the addition of NADP analog or NAD diphosphate.

Distribution of NAD phosphorylating activities, NAD kinase and phosphoryl transferring activity with *p*-NPP (designated as phosphotransferase), were discussed. NAD kinase activity was rather widely distributed in genera of bacteria, whereas phosphotransferase activity was limited to a few genera. *P. mirabilis* showed striking activity of phosphotransferase.

Purification of phosphotransferase of *P. mirabilis* was carried out and partially purified enzyme preparation was obtained through treatment with protamine sulfate, precipitation with polyethylene

glycol and separation with DEAE-Sephadex column chromatography. Enzyme preparations in each purification steps showed both phosphatase and phosphotransferase activities. This result suggest that phosphoryl transfer and hydrolysis of *p*-NPP may be catalyzed by the same enzyme. General properties of the enzyme activity were studied. Optimal pH for phosphoryl transfer was below 4.0. Cupric ion stimulated both activities of phosphotransferase and phosphatase. Not only *p*-NPP, but also nucleotides were available as phosphoryl donor. Furthermore, nucleosides and nucleotides as well as pyridine coenzymes were phosphorylated. These results may indicate the possibility that a transferase is involved in the phosphorylation of NAD.

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